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CONTROL, PRODUCTION AND CHARACTERIZATION OF
EXTRACELLULAR ENZYMES OF MICROCOC'CUS SODONENSIS

by



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A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Control, Production and Characterization of Extracellular Enzymes of Micrococcus sodonensis" submitted by Cecily Mills in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

Micrococcus sodonensis has been shown to produce several extracellular enzymes: an alkaline phosphatase, at least two forms of diesterase, a 5'-nucleotidase, and an alkaline proteinase. The almost quantitative release of the enzymes into the culture medium during logarithmic growth under all the various culture conditions indicates that these enzymes are extracellular enzymes. Inorganic phosphate represses the production of the alkaline phosphatase in synthetic as well as in complex media, whereas, the repression of the production of the diesterase and the 5'-nucleotidase by inorganic phosphate is partly reversed by the addition of organic nutrients to the culture media. The addition of organic nitrogen as casamino acids to the synthetic medium results in the repression of the alkaline phosphatase without affecting the other enzymes. The amount of proteinase produced per mg dry weight of cells is the same regardless of the culture conditions used. A mutant strain of M. sodonensis has an altered production of diesterase but the mutation does not affect the production of the other extracellular enzymes. These results suggest that the extracellular enzymes of M. sodonensis are not produced in a pleiotropic fashion. Extracellular high molecular

weight carbohydrate is produced by M. sodonensis in synthetic medium. The carbohydrate fraction appears to contain glycoprotein.

The alkaline phosphatase has been purified to homogeneity as judged by polyacrylamide gel electrophoresis and sedimentation velocity analysis, and was shown to have a broad substrate specificity, hydrolyzing a wide variety of phosphorylated substrates at comparable rates. Calcium is required for stabilization of the enzyme as well as for expression of catalytic activity. The Michaelis constant for p-nitrophenyl phosphate is 2.4×10^{-5} M. Inorganic phosphate is a competitive inhibitor of the hydrolysis reaction when p-nitrophenyl phosphate is employed as substrate. The alkaline phosphatase of M. sodonensis possesses transphosphorylase activity as demonstrated by the ability of the enzyme to transfer phosphate from pyrophosphate or AMP to glucose to form glucose-6-phosphate.

The kinetics of the 5'-nucleotidase of M. sodonensis were studied and it appears that both products of the hydrolytic reaction, the nucleoside and the inorganic phosphate, interact with the enzyme. The binding of these products to the enzyme was detected by equilibrium dialysis of the nucleoside with the enzyme, by elution of ^{32}P -inorganic phosphate with the enzyme on gel filtration, by protection from inactivation, by heat or by protein reagents, afforded by both of the hydrolysis products. The kinetic data suggest an apparent Ordered Uni Bi mechanism for the hydrolytic reaction.

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TABLE OF CONTENTS

	Page
ABSTRACT	iii
ACKNOWLEDGEMENTS	v
LIST OF TABLES	ix
LIST OF FIGURES	x
LIST OF ABBREVIATIONS	xv
INTRODUCTION	1
MATERIALS AND METHODS	23
I. Organism and Growth Conditions . . .	23
II. Culture Media	23
III. Control Studies	23
IV. Enzyme Production	23
V. Enzyme Purification	26
(1) Ion Exchange Chromatography .	26
(2) Gel Filtration	26
(3) Ammonium Sulfate Fractionation	27
(4) Ultracentrifugation	27
(5) Disc Gel Electrophoresis . .	28
VI. Preparation of Cell-Free Extracts. .	29
VII. Enzyme Assays	29
(1) Alkaline Phosphatase	29
(2) 5'-Nucleotidase	30
(3) Diesterase	31
(4) Transphosphorylase	31
(5) Proteinase	32

	Page
VIII. Isolation of Mutants	vii 32
IX. Analytical Methods	33

EXPERIMENTAL RESULTS

I. Multiplicity and Control of Extracellular Enzymes Produced by <u>M. sodonensis</u>	34
(1) The Extracellular Enzymes of <u>M. sodonensis</u>	34
(a) Diesterases	34
(b) 5'-Nucleotidase	38
(c) Proteinase	40
(d) Alkaline Phosphatase	41
(2) Extracellular Nature of the Enzymes	41
(3) Effect of Growth Medium on Production of Extracellular Enzymes	45
(4) Control of Enzyme Production by a Mutant Strain	49
(5) Control of Proteinase Production	51
(6) Production of Extracellular Carbohydrate	51
II. The Alkaline Phosphatase of <u>M. sodonensis</u>	59
(1) Purification	59
(2) Substrate Specificity	65
(3) pH Optimum	69
(4) Cation Requirements	69
(5) Kinetic Analyses	77
(6) Transphosphorylase Activity	82
(7) Enzyme Inhibition	85

	Page
III. Kinetic Study of the 5'-Nucleotidase. . .	87
(1) Purification	87
(2) Binding of Products to the Enzyme. .	87
(3) Product Inhibition by P_i	95
(4) Product Inhibition by Nucleosides. .	99
(5) Effect of Alternative Substrates on AMP Hydrolysis	109
DISCUSSION	115
BIBLIOGRAPHY	127

LIST OF TABLES

TABLE		Page
I.	COMPOSITION OF CULTURE MEDIA USED FOR <u>M. SODONENSIS</u> CONTROL STUDIES	24
II.	EFFECT OF ALTERATION OF GROWTH MEDIA ON EXTRACELLULAR ENZYME PRODUCTION BY <u>M. SODONENSIS</u>	47
III.	EFFECT OF ALTERATION OF GROWTH MEDIA ON PROTEINASE PRODUCTION BY <u>M. SODONENSIS</u> . .	53
IV.	ALKALINE HYDROLYSIS OF EXTRACELLULAR CARBOHYDRATE FRACTION OF <u>M. SODONENSIS</u> . .	57
V.	AMMONIUM SULFATE FRACTIONATION OF <u>M. SODONENSIS</u> ALKALINE PHOSPHATASE	62
VI.	PURIFICATION OF <u>M. SODONENSIS</u> ALKALINE PHOSPHATASE	63
VII.	SUBSTRATE SPECIFICITY OF <u>M. SODONENSIS</u> ALKALINE PHOSPHATASE	68
VIII.	EFFECT OF DIVALENT CATIONS ON <u>M. SODONENSIS</u> ALKALINE PHOSPHATASE ACTIVITY	72
IX.	EFFECT OF CHELATING AGENTS ON <u>M. SODONENSIS</u> ALKALINE PHOSPHATASE ACTIVITY	73
X.	EFFECT OF SOME ENZYME INHIBITORS ON <u>M. SODONENSIS</u> ALKALINE PHOSPHATASE ACTIVITY	86
XI.	EFFECT OF SOME ENZYME INHIBITORS ON <u>M. SODONENSIS</u> 5'-NUCLEOTIDASE ACTIVITY . .	91

LIST OF FIGURES

FIGURE		Page
1.	ELUTION PROFILE OF <u>M. SODONENSIS</u> EXTRACELLULAR ENZYMES FROM DEAE-CELLULOSE COLUMN	35
2.	POLYACRYLAMIDE GEL ELECTROPHORESIS OF VARIOUS PREPARATIONS OF <u>M. SODONENSIS</u> DIESTERASE	37
3.	RECHROMATOGRAPHY ON DEAE-CELLULOSE OF <u>M. SODONENSIS</u> 5'-NUCLEOTIDASE	39
4.	GROWTH RESPONSE AND ENZYME PRODUCTION BY <u>M. SODONENSIS</u> IN SYNTHETIC MEDIUM	43
5.	GROWTH RESPONSE AND ENZYME PRODUCTION BY <u>M. SODONENSIS</u> IN TCS BROTH.	44
6.	POLYACRYLAMIDE GEL ELECTROPHORESIS OF CULTURE SUPERNATANTS OF <u>M. SODONENSIS</u> GROWN IN SYNTHETIC MEDIUM WITH AND WITHOUT ADDED P_i	50
7.	POLYACRYLAMIDE GEL ELECTROPHORESIS OF CULTURE SUPERNATANTS OF WILD TYPE AND MUTANT STRAIN 2-B. OF <u>M. SODONENSIS</u> GROWN IN SYNTHETIC MEDIUM.	52
8.	GROWTH RESPONSE AND HEXOSAMINE PRODUCTION BY <u>M. SODONENSIS</u> IN SYNTHETIC MEDIUM	56
9.	ELUTION PROFILE OF <u>M. SODONENSIS</u> ALKALINE PHOSPHATASE FROM SEPHADEX G-200	61
10.	SCHLIEREN PATTERN OF <u>M. SODONENSIS</u> ALKALINE PHOSPHATASE	64
11.	GEL ELECTROPHORESIS OF PURIFIED <u>M. SODONENSIS</u> ALKALINE PHOSPHATASE	66

12.	MOLECULAR WEIGHT DETERMINATION OF <u>M. SODONENSIS</u> ALKALINE PHOSPHATASE BY THE GEL FILTRATION METHOD	67
13.	pH ACTIVITY CURVE OF <u>M. SODONENSIS</u> ALKALINE PHOSPHATASE	70
14.	EFFECT OF 1.7×10^{-3} M Ca^{2+} ON THE RATE OF INACTIVATION OF <u>M. SODONENSIS</u> ALKALINE PHOSPHATASE AT ROOM TEMPERATURE . . .	74
15.	EFFECT OF 1.7×10^{-3} M Ca^{2+} ON THE RATE OF INACTIVATION OF <u>M. SODONENSIS</u> ALKALINE PHOSPHATASE AT 55°	75
16.	EFFECT OF 1.7×10^{-3} M Ca^{2+} ON THE STABILITY OF <u>M. SODONENSIS</u> ALKALINE PHOSPHATASE AT VARIOUS TEMPERATURES. . .	76
17.	EFFECT OF ENZYME CONCENTRATION ON ACTIVITY OF <u>M. SODONENSIS</u> ALKALINE PHOSPHATASE	78
18.	INHIBITION BY P_i OF THE HYDROLYSIS OF PNPP BY <u>M. SODONENSIS</u> ALKALINE PHOSPHATASE	79
19.	RELOT OF SLOPES OBTAINED IN FIGURE 18 AGAINST P_i CONCENTRATION	80
20.	INHIBITION BY P_i OF AMP HYDROLYSIS BY <u>M. SODONENSIS</u> ALKALINE PHOSPHATASE . . .	81
21.	TRANSPHOSPHORYLASE ACTIVITY OF <u>M. SODONENSIS</u> ALKALINE PHOSPHATASE	83
22.	EFFECT OF INCREASING CONCENTRATIONS OF TRIS ON <u>M. SODONENSIS</u> ALKALINE PHOSPHATASE ACTIVITY	84
23.	EFFECT OF P_i ON HEAT INACTIVATION OF <u>M. SODONENSIS</u> 5'-NUCLEOTIDASE AT 65° . . .	89
24.	EFFECT OF ADENOSINE ON HEAT INACTIVATION OF <u>M. SODONENSIS</u> 5'-NUCLEOTIDASE AT 71° . . .	90
25.	SCATCHARD PLOT SHOWING BINDING OF ^{14}C -ADENOSINE TO <u>M. SODONENSIS</u> 5'-NUCLEOTIDASE	94

26.	ELUTION PROFILE OF <u>M. SODONENSIS</u> 5'-NUCLEOTIDASE AND $^{32}\text{P}_i$ FROM SEPHADEX G-50	96
27.	LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF P_i ON <u>M. SODONENSIS</u> 5'-NUCLEOTIDASE ACTIVITY AT LOW SUBSTRATE CONCENTRATIONS	97
28.	LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF P_i ON <u>M. SODONENSIS</u> 5'-NUCLEOTIDASE ACTIVITY AT HIGHER SUBSTRATE CONCENTRATIONS	98
29.	LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF ADENOSINE ON <u>M. SODONENSIS</u> 5'-NUCLEOTIDASE ACTIVITY	100
30.	REPLOTS OF INTERCEPTS AND SLOPES FROM FIGURE 29 AGAINST ADENOSINE CONCENTRA- TION	101
31.	RELOT OF RECIPROCAL OF SLOPES AND INTERCEPTS FROM FIGURE 29 AGAINST RECIPROCAL OF ADENOSINE CONCENTRATION.	103
32.	LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF CYTIDINE AND DEOXYCYTIDINE ON THE HYDROLYSIS OF CMP AND dCMP RESPECTIVELY BY <u>M. SODONENSIS</u> 5'-NUCLEOTIDASE	104
33.	LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF URIDINE AND DEOXYURIDINE ON THE HYDROLYSIS OF UMP AND dUMP RESPECTIVELY BY <u>M. SODONENSIS</u> 5'-NUCLEOTIDASE	105
34.	LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF ADENOSINE AND DEOXYADENOSINE ON THE HYDROLYSIS OF AMP AND dAMP RESPECTIVELY BY <u>M. SODONENSIS</u> 5'-NUCLEOTIDASE	106

35.	LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF GUANOSINE AND DEOXYGUANOSINE ON THE HYDROLYSIS OF GMP AND dGMP RESPECTIVELY BY <u>M. SODONENSIS</u> 5'-NUCLEOTIDASE	107
36.	LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF INOSINE AND XANTHOSINE ON THE HYDROLYSIS OF IMP AND XMP RESPECTIVELY BY <u>M. SODONENSIS</u> 5'-NUCLEOTIDASE	108
37.	LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF UMP AND dUMP OF THE HYDROLYSIS OF ^{14}C -AMP BY <u>M. SODONENSIS</u> 5'-NUCLEOTIDASE	110
38.	LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF GMP AND dGMP OF THE HYDROLYSIS OF ^{14}C -AMP BY <u>M. SODONENSIS</u> 5'-NUCLEOTIDASE	111
39.	LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF CMP AND dCMP ON THE HYDROLYSIS OF ^{14}C -AMP BY <u>M. SODONENSIS</u> 5'-NUCLEOTIDASE	112
40A.	LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF dAMP ON THE HYDROLYSIS OF ^{14}C -AMP BY <u>M. SODONENSIS</u> 5'-NUCLEOTIDASE.	113
40B.	LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF XMP AND IMP OF THE HYDROLYSIS OF ^{14}C -AMP BY <u>M. SODONENSIS</u> 5'-NUCLEOTIDASE	113

LIST OF ABBREVIATIONS

A	Absorbance
AMP, CMP, UMP, GMP, IMP, XMP	5'-Monophosphates of adenosine, cytidine, uridine, guanosine, inosine, xanthosine, respectively
dAMP, dCMP, etc.	5'-Monophosphates of deoxyadenosine, deoxycytidine, etc.
DEAE	Diethylamino ethyl
EDTA	Ethylenediaminetetraacetate
Hepes	N-2-hydroxyethylpiperazine-N-2- ethanesulfonic acid
K_i	Inhibitor constant
K_m	Michaelis constant
NADP^+ , NADPH	Nicotinamide-adenine dinucleotide phosphate, oxidized and reduced forms
P_i	Orthophosphate (inorganic)
PP_i	Pyrophosphate (inorganic)
PNPP	<u>p</u> -Nitrophenyl phosphate
p.s.i.	Pounds per square inch
TCA	Trichloroacetic acid
TCS	Trypticase soy (Baltimore Biological Laboratories, Inc.)
TEAE	Triethylamino ethyl
Tris	Tris (hydroxymethyl) aminomethane

INTRODUCTION

Among the microbial enzymes referred to as extracellular or extraprotoplasmic, three types which have received considerable study are proteinases, alkaline phosphatases, and nucleases. The following discussion is restricted chiefly to the proteinases and the alkaline phosphatases.

Extracellular proteinases are produced by a large number of microorganisms. These enzymes usually fall into two broad categories: alkaline proteinases and neutral proteinases. Most of the neutral proteinases are metallo-enzymes, inhibited by metal chelating agents but not by diisopropyl fluorophosphate (DFP); whereas most of the alkaline proteinases are serine-enzymes completely inhibited by DFP but not affected by EDTA. The neutral proteinases possess specificity to the amino residue which involves the amino group to be hydrolyzed and the alkaline proteinases possess specificity to the amino acid residue which contains the carboxyl group to be hydrolyzed (Moriyama, 1967).

Bacillus subtilis produces both types of extracellular proteinases. The alkaline proteinase of B. subtilis is called subtilisin (Guntelberg and Ottesen, 1954; Ottesen,

and Svendsen, 1970) (EC 3.4.4.16, with the recommended trivial name subtilopeptidase A). In addition, B. subtilis produces a neutral proteinase (McConn, Tsuru, Yasunobu, 1964) which contains Zn^{2+} as the prosthetic group. Another subtilisin-like proteinase was isolated by Rappaport et al (1965), from a transformable strain of B. subtilis. This proteinase has an unusually high molecular weight around 166,000 and it consists probably of several subunits. B. licheniformis also produces both a neutral and an alkaline proteinase (Hall et al, 1966). Only the neutral proteinase, a zinc-containing metalloenzyme, is produced by B. megaterium. It was shown to be similar in specificity to the neutral proteinase of B. subtilis (Millet and Acher, 1968; Keay, et al, 1971) and to thermolysin, the neutral proteinase of B. thermoproteolyticus (Matsubara et al, 1966). B. cereus produces only a neutral proteinase (Levisohn and Aronson, 1967).

Extracellular proteinases have been studied with Coccus P, a Sarcina closely related to Micrococcus lysodeikticus, which produces two proteolytic enzymes, one which is excreted into the medium during the exponential phase of growth and the other which is loosely associated with the cells and is released upon spheroplast formation, by washing with saline or even by the culture medium itself (Sarner, et al, 1971).

A neutral proteinase, a Zn^{2+} - metalloenzyme, from

a Serratia species has been isolated and characterized (Miyata, Tomada, and Isono, 1971). In addition, proteinase production by many other bacteria and fungi have been reported and studied (Hagihara, 1960; Pollock, 1962; Davies, 1963; Ahearn, Meyers, and Nichols, 1968).

In most cases proteinases are produced during post-exponential growth phase, although some species do produce the enzyme during exponential growth when grown under appropriate conditions (Chaloupka and Kreckova, 1966; Millet and Aubert, 1969). Kinetic studies carried out with B. licheniformis (Bernlohr, 1964), B. subtilis (Coleman, 1967; Michel, 1966), and B. cereus (Levisohn and Aronson, 1967) showed this activity to increase rapidly at the end of growth as a result of de novo synthesis, rather than as a result of delayed excretion or activation of a preformed enzyme. On the other hand, the proteinase of Coccus P is produced during the exponential phase of growth (Sarner, et al., 1971).

Some bacterial proteinases are known to be secreted as zymogens. Streptococcal proteinase zymogen can be converted to the active enzyme by proteolysis followed by reduction; the proteolysis can be achieved either by the action of trypsin or the streptococcal proteinase itself. The reducing activity of the streptococcal cell walls initiates the transformation and is responsible for activating by reduction part or all of the proteinase ultimately found (Liu and Elliott, 1965). Likewise, the zymogen of the proteinase

of Coccus P is activated by trypsin or by the proteinase itself in an autocatalytic reaction (Gorini and Lanzavecchia, 1954). The authors suggest that since the zymogen is activated more rapidly at higher temperatures and at higher pH's, other bacteria may also form their proteolytic enzymes as zymogens but that, in the usual growth conditions, it is immediately converted to the active form.

Calcium plays an important role in the stabilization of many proteinases (Gorini, 1950). The extracellular proteinase of Coccus P undergoes rapid autodigestion in the absence of Ca^{2+} (Sarner, 1971; Bissell et al, 1971). Ca^{2+} is required for activity of the B. cereus (Levisohn and Aronson, 1967) and B. megaterium (Millet, Acher and Aubert, 1969) proteinases, and has a stabilizing effect on the neutral proteinase of B. subtilis (McConn et al, 1964) and of Micrococcus caseolyticus (Desmazeaud and Hermier, 1968). Many proteases lack disulfide bonds and Ca^{2+} electrostatic bridges may replace the missing disulfide bridges in securing proper folding. Once the enzyme is stabilized by Ca^{2+} , it is no longer susceptible to proteolytic attack (Sarner, et al, 1971). This has also been found to be the case with other enzymes; for instance, Ca^{2+} is necessary for maintaining α -amylase (Stein and Fischer, 1960) and trypsin (Gorini and Felix, 1953) in a form which is resistant to proteolytic attack.

In some microorganisms, proteinase production is

repressed by the presence of amino acids. This is the case with the proteinase of B. cereus (Levisohn and Aronson, 1967; Neumark and Citri, 1962), B. megaterium (Chaloupka et al, 1963), B. subtilis (May and Elliott, 1968), P. lachrymans (Keen and Williams, 1967), Arthrobacter sp. (Hofsten and Tjeder, 1965), and others. In some cases one or a certain combination of 2 or 3 amino acids results in maximum repression of proteinase production (Chaloupka et al, 1963; May and Elliott, 1968); whereas in others, all the amino acids are required for maximum repression (Millet, Acher and Aubert, 1969).

On the other hand, the proteinase production of B. pyocyaneus is roughly parallel with growth. It is not dependent on the ingredients of the nutrient medium (Wilson, 1930). The Pseudomonadaceae synthesize the enzyme mostly during growth in medium containing an organic source of nitrogen (Keen and Williams, 1967). Likewise, the presence of peptides or proteins is necessary for enzyme production in the case of Arthrobacter and some micrococci (Din et al, 1969). The extracellular proteinase of Micrococcus freundenreichii is induced by amino acids (McDonald and Chambers, 1966). The authors propose that the induction ("end-product induction") of extracellular proteinase by amino acids with this Micrococcus and its repression by metabolizable carbon sources suggest that the primary function of this proteinase is to ensure a supply of carbon for growth rather than a

supply of amino acids for synthetic purposes.

The production of most proteinases is subject to catabolite repression. Increases in the concentration of glucose or acetate (carbon source) had a repressive effect on the proteinases of B. megaterium (Millet, Acher and Aubert, 1969), B. cereus (Levisohn and Aronson, 1967), B. licheniformis (Bernlohr, 1964; Bernlohr and Clark, 1971). In the genus Bacillus, protease production appears to be closely linked to the capacity to sporulate (Schaeffer, 1969; Aronson, Angelo, and Holt, 1971). Relief from metabolic repression might be the common origin of sporulation and the production of proteinase. From a rapidly metabolized growth substrate, and in the presence of an utilizable nitrogen source, metabolites formed may repress the synthesis of both the proteinase and some early sporulation-specific enzyme.

Several species of microorganisms produce extra-cellular alkaline phosphatases. An alkaline phosphatase has been isolated from Escherichia coli using the supernatant fraction obtained during spheroplast formation with lysozyme and EDTA in an osmotically protective medium (Malamy and Horecker, 1961, 1964a). The lysozyme-EDTA treatment released 93% of the alkaline phosphatase activity whereas intracellular enzymes such as glucose-6-phosphate dehydrogenase, glutamic dehydrogenase, and β -galactosidase were retained (Malamy and Horecker, 1964a). Alkaline phosphatase is also released quantitatively from E. coli by osmotic shock (Neu

and Heppel, 1965). The procedure consists of incubation of the cells in a hyperosmolar solution of sucrose and EDTA followed by a sedimentation of the cells and exposure to either cold water or a dilute magnesium solution.

A number of other degradative enzymes are specifically released from exponentially growing E.coli by osmotic shock or lysozyme-EDTA treatment. They include cyclic phosphodiesterase, 5'-nucleotidase, acid hexose phosphatase (Neu and Heppel, 1965), acid phenyl phosphatase (Neu, 1967), thymidine phosphorylase (Kammen, 1967), adenosine diphosphate-glucose pyrophosphatase (Melo and Glaser, 1966), the ribonucleic acid-inhibited endonuclease (Nossal and Heppel, 1966; Anraku, 1964), diphosphate sugar hydrolase (Glaser, Melo, and Paul, 1967), asparaginase II (Cedar and Schwartz, 1968), and a phosphate-binding protein probably involved in P_i transport (Medveczky and Rosenberg, 1970). The group of enzymes set free by osmotic shock or during the formation of spheroplasts (with EDTA and lysozyme) is similar to the enzymes found in mammalian lysosomes (Neu and Chou, 1967). Most of the enzymes released are degradative and concerned with phosphate, nucleotide and sugar metabolism.

The lysozyme-EDTA treatment and osmotic shock are also effective in the selective release of enzymes from some other gram-negative organisms. Among the Enterobacteriaceae, osmotic shock causes the release of a group of degradative enzymes from all organisms except members of Proteus and

Providencia groups (Neu and Chou, 1967). In Pseudomonas aeruginosa simple washing in 0.2 M Mg^{2+} effectively releases 100% of the alkaline phosphatase while the cells remain viable and actively motile (Cheng et al, 1970a). Ca^{2+} also released a substantial portion of the alkaline phosphatase. Tris buffer, monovalent ions, 20% sucrose, and water released only part of the total enzyme from the cells. Identical treatment of E. coli cells did not yield alkaline phosphatase (Cheng et al, 1970a).

With Spirillum itersonii, treatment of the cells with Tris-EDTA, without osmotic shock, resulted in the quantitative release of alkaline phosphatase and ribonuclease (Garrard, 1971). Similar treatment of E. coli cells did not result in significant release of periplasmic enzymes but did result in a transient nonspecific increase in permeability, loss of lipopolysaccharide material and a depletion of the nucleotide pool (Garrard, 1971; Buttin and Kornberg, 1966; Leive, 1968; Neu, Ashman, and Price, 1966; Tucker and White, 1970).

The selective release of alkaline phosphatase and other enzymes from E. coli and other organisms by shock treatment or lysozyme-EDTA treatment suggests that these enzymes are external to the cytoplasmic membrane. These are not extracellular enzymes, because they are not released into the medium during growth of the bacteria. Neu and Chou (1967) suggest that this group of enzymes exists bound to

the cytoplasmic membrane of Gram negative cells and in Gram positive cells with their less complicated, less hydrophobic cell wall, these enzymes escape as extracellular enzymes.

Gram negative cells treated with lysozyme alone retain their rod like structure and fail to release any enzymes, whereas, at the moment EDTA strikes the lysozyme-treated cell, it alters its shape to become a spheroplast and releases the enzymes (Birdsell and Cota-Robles, 1967). These facts suggest that these groups of enzymes are loosely bound to the cytoplasmic membrane through the mediation of divalent cations (Neu and Chou, 1967).

Malamy and Horecker, (1964a, b) also suggest that alkaline phosphatase is localized in the periplasmic space. This is based on their measurement of the activity of intact cells versus phosphorylated compounds. Many phosphate esters for which no transport system into the cell is known are split by the intact cells. Therefore the enzyme must be localized on the surface. The apparent K_m for hydrolysis of PNPP and other substrates is higher for intact cells than for cell extracts. This suggests a passive barrier between the substrate and the cell membrane.

Cytochemical localization of alkaline phosphatase in E. coli at the electron microscope level confirmed the periplasmic location of the enzyme. Electron dense material is deposited between the outer membrane and the plasma membrane (Malamy and Horecker, 1964a; Brockman and Heppel, 1968;

Done et al, 1965). Repressed cells or cells to which no β -glycerophosphate had been added failed to show such electron-dense material. Immunological studies showed that the enzyme is not located at the cell surface (Schlesinger and Olson, 1968).

In the case of the alkaline phosphatase from Pseudomonas aeruginosa which is released by high Mg^{2+} concentrations, Cheng et al (1970a) suggest that the enzyme is bound by electrostatic forces which probably involve linkages, mediated by Mg^{2+} , between anionic centers in the enzyme and components of the cell external to the cytoplasm. The reaction of high Mg^{2+} may proceed by way of competition with the anionic centers, resulting in the removal of the enzyme. The conditions necessary for the removal of the alkaline phosphatase from P. aeruginosa exert little or no effect upon the cell, since neither viability nor growth rates are affected by this treatment. The in vivo binding site for this periplasmic alkaline phosphatase may be the lipopolysaccharide of the inner double track layer of the cell wall (Cheng, Ingram, and Costerton, 1970b, 1971). Cytochemical localization of this enzyme at the electron microscope level also confirmed the periplasmic location of the enzyme.

Spheroplasts of E. coli produced by penicillin and D-cycloserine released only a small portion of the alkaline phosphatase activity. When the penicillin-produced sphero-

plasts were lysed with water, the enzyme activity was completely soluble.

The alkaline phosphatase of E. coli and of most other Gram negative organisms is a surface-bound enzyme situated outside of the main permeability barrier of the cell.

The localization satisfies the following criteria of Pollock (1962) for surface-bound enzymes:

- 1) All alkaline phosphatase activity sediments with the cells during centrifugation. The enzyme is not found in any substantial amounts in the culture medium.

- 2) The enzyme is quantitatively released from the cells into the medium when lysozyme spheroplasts are formed in a medium of high osmotic pressure.

- 3) Experiments with phosphatase-containing cells have shown that impermeable substrates such as glucose-6-phosphate are rapidly hydrolyzed by intact cells with the release of most of the phosphate into the medium (Malamy and Horecker, 1964a).

The enzyme is not an integral component of the cell wall. When the cell wall preparations are made, no phosphatase activity is found associated with the particulate fraction; it is completely solubilized (Malamy and Horecker, 1964a).

The localization of the alkaline phosphatase of various Gram positive organisms differs from species to species and even among strains of the same species. In

B. subtilis, Takada and Tsugita (1967) found the alkaline phosphatase to be particulate (i.e., probably membrane bound); however, in another strain of B. subtilis, it is an exoenzyme (Cashel and Freeze, 1964). Wood and Tristram (1970) found 15-30% of the total alkaline phosphatase activity in the medium. This extracellular enzyme was largely particulate. The conversion of both B. subtilis and B. megaterium to protoplasts did not result in the liberation of the cell-bound alkaline phosphatase. Lysis of the protoplasts revealed that 90% of the total enzyme activity associated with the cells resided in the protoplasmic membrane fraction. The enzyme was solubilized by extraction of the membranes or of intact cells with molar concentration of various salts. The same diversity of localization of alkaline phosphatase was found in B. licheniformis. Chesbro and Lampen (1968) found that a mesophilic strain of B. licheniformis liberated 95% of the alkaline phosphatase into the culture medium. In another strain of the same organism, Hulett-Cowling and Campbell (1971a) found that the alkaline phosphatase was cell-bound and not liberated on spheroplast formation or by osmotic or cold shock. When the cells were lysed with lysozyme, the enzyme activity was associated with the membrane fraction and could only be solubilized by treatment with 1M Mg^{2+} . Histochemical localization of alkaline phosphatase in B. subtilis showed that the enzyme occurs mainly in the peripheral membrane-polyribosome

complex (Ghosh, Wouters, and Lampen, 1970).

Most of the alkaline phosphatases studied so far have proven to be metalloenzymes. Metal chelating agents have been found to inhibit E.coli alkaline phosphatase (Garen and Levinthal, 1960; Heppel, Harkness and Hilmoe, 1962; Plocke, Levinthal, and Vallee, 1962). The latter observed that alkaline phosphatase from E.coli contained at least two gram atoms of zinc per mole of enzyme. Later investigators determined that four gram atoms of zinc were present per mole of enzyme (Simpson and Vallee, 1968; Petitclerc et al, 1970). Two sites of type I bind zinc tightly, while the two sites of type II bind it more loosely. Only the Zn^{2+} -phosphatase with four gram atoms of zinc per mole of protein is an active enzyme (Petitclerc, et al, 1970).

The inhibition of enzymatic activity by a number of metal-binding agents followed the order expected on the basis of the known stability constants of their soluble complexes with zinc ions. The addition of cobalt in stoichiometric amount to the apoenzyme generates a catalytically active cobalt enzyme (Plocke and Vallee, 1962) whose enzymatic activity is about 12% of that of the native enzyme (Gottesman, Simpson, and Vallee, 1969). The Cu^{2+} -alkaline phosphatase has also been prepared (Lazdunski et al, 1970) and found considerably less active than the Zn^{2+} - and Co^{2+} -enzymes. Both the Cu^{2+} - and the Co^{2+} -enzymes have some

distinctive characteristics distinguishing them from each other and from the Zn^{2+} -enzyme (Lazdunski and Lazdunski, 1968; Lazdunski, Petitclerc, and Lazdunski, 1969).

The alkaline phosphatase from Aspergillus nidulans is also inhibited by chelating agents and the metal bound by the enzyme is thought to be Mg^{2+} (Dorn, 1968). The non-repressible alkaline phosphatase of Neurospora crassa is also believed to be an Mg^{2+} -enzyme (Kuo and Blumenthal, 1961) whereas the absolute metal requirement for the repressible enzyme from the same organism remains in doubt (Kadner and Nyc, 1969).

With E. coli alkaline phosphatase, it was noted that below pH 3.5, the sedimentation coefficient changed from 6.15 to 2.6 (Malamy and Horecker, 1964b). A combined treatment with urea and thioglycollic acid also resulted in loss of activity and the denatured material now had a molecular weight of 40,000, half of the molecular weight assigned to the native enzyme (Garen and Levinthal, 1960; Rothman and Byrne, 1963). Analysis of the peptides produced by tryptic digestion of the protein demonstrated that the enzyme is composed of two subunits with apparently identical amino acid composition (Rothman and Byrne, 1963). When the E. coli gene for alkaline phosphatase production was introduced into Serratia marcescens, which produces a distinct alkaline phosphatase, both enzymes were produced and also, by intergeneric hybridization of the subunits of the two

distinct enzymes, a hybrid enzyme was produced composed of one monomer of the E. coli type with one monomer of the S. marcescens type (Levinthal, Signer and Fetherolf, 1962). Neutralization of the acidified E. coli monomer units leads to refolding of the individual chains followed by dimerization to an enzymatically active protein (Schlesinger and Levinthal, 1963). The rate of reactivation can be increased several fold with the addition of metals to the reaction mixture, Zn^{2+} being the most effective (Schlesinger and Barrett, 1965). EDTA prevents the reassociation of the monomers. Removal of Zn^{2+} from the native enzyme does not result in dissociation of the enzyme into subunits (Schlesinger and Barrett, 1965). Spheroplasts of E. coli produce the same relative amount of alkaline phosphatase protein as do intact cells but the protein appears as inactive subunits in the culture medium. Therefore, the subunits not the dimers pass through the cell membrane (Schlesinger, 1968). Transport of this enzyme to the periplasmic space as an inactive subunit would provide one means of protecting the bacterial cell from the action of an active alkaline phosphatase in its cytoplasm. Monomers are present when the cell is broken open and are associated with a particulate fraction resistant to ribonuclease (Torriani, 1968).

The repressible alkaline phosphatase in Neurospora

crassa can also be dissociated by guanidine-mercaptoethanol into subunits having a molecular weight equal to one half of the molecular weight determined for the native enzyme (Kadner, Nyc, and Brown, 1968). Similarly, treatment of the alkaline phosphatase of Bacillus licheniformis with guanidine-HCl, urea, or dilute HCl yields a subunit with a molecular weight equal to one half that of the active enzyme (Hulett-Cowling and Campbell, 1971b). The alkaline phosphatase of Pseudomonas aeruginosa also consists of two subunits of molecular weight of 60,000 as compared to a molecular weight of 125,000 for the active phosphatase. The low pH of the culture causes the dissociation of the alkaline phosphatase into subunits as it is released into the culture medium (Cheng, Costerton, and Ingram, 1971).

E. coli alkaline phosphatase was found to hydrolyze almost all of the phosphomonoesters tested (Garen and Levinthal, 1960; Torriani, 1960; Heppel, Harkness and Hilmo, 1962). The organic moieties of the phosphomonoester substrates have only a small effect on the rate of enzymic activity (Garen and Levinthal, 1960; Heppel, Harkness and Hilmo, 1962). Earlier reports claimed that inorganic pyrophosphate and nucleoside triphosphates were not cleaved by E. coli alkaline phosphatase (Garen and Levinthal, 1960; Torriani, 1960); however, these were subsequently found to be substrates (Heppel, Harkness and Hilmo, 1962; Fernley and Walker, 1967). Besides having orthophosphoric monoester

phosphohydrolase activity, E. coli alkaline phosphatase has been shown to catalyze the transfer of a phosphoryl group of inorganic pyrophosphate to the hydroxyl group attached to carbon atom 6 of glucose (Anderson and Nordlie, 1967). Transphosphorylation of Tris, ethanolamine, and glycerol by the E. coli alkaline phosphatase accounts for the increase in enzyme activity noted with increasing concentrations of these acceptors (Dayan and Wilson, 1964; Wilson, Dayan and Cyr, 1964). The transphosphorylation reaction has not been studied in the alkaline phosphatases isolated from other microbial sources. It occurs in numerous mammalian alkaline phosphatases (Cox, Gilbert, and Griffin, 1967; Morton, 1959).

The reaction mechanism for alkaline phosphatases has been proposed to be like the one described for microsomal glucose 6-phosphatase by Arion and Nordlie (1964). This mechanism involves successively (a) formation of a binary enzyme-phosphoryl-substrate complex, (b) a dissociation leaving a phosphoryl-enzyme intermediate, and (c) transfer of the phosphoryl group from enzyme either to water (hydrolysis) or to glucose or other acceptor (phosphotransferase) (Wilson, Dayan and Cyr, 1964; Anderson and Nordlie, 1967). There is evidence for a phosphoryl enzyme intermediate. E. coli alkaline phosphatase becomes phosphorylated by either substrate or phosphate (Schwartz and Lipmann, 1961; Engström, 1962). Phosphorylated serine was isolated and it

was concluded that of the thirty-three serine molecules known to be present in the E. coli alkaline phosphatase, only a particular one reacts with the phosphate. Phosphorylation is maximal at pH 4-5 (where enzymatic activity is minimal) (Schwartz and Lipmann, 1961; Engström, 1962). At pH 9, where the relative velocity of the enzyme is maximal, phosphorylation is minimal (Schwartz, 1963), yet phosphate is firmly bound non-covalently (Levinthal et al, 1962; Lazdunski, Petitclerc, Chappelet, and Lazdunski, 1969) and strongly inhibits the enzyme (Garen and Levinthal, 1960). Since the phosphate group of the substrate competes with P_i for the specific serine in the enzyme, the phosphate-binding serine is most likely in the active center of the enzyme (Schwartz and Lipmann, 1961). The incorporation of phosphate by a serine residue of alkaline phosphatase has been noted in a number of other alkaline phosphatases and the peptide sequence asp-serP-ala is common to E. coli, Serratia marcescens, and calf intestinal mucosa alkaline phosphatase (Milstein, 1964; Zwaig and Milstein, 1964). Metal chelating agents inhibit incorporation suggesting the participation of the metal ion in the incorporation reaction (Engström, 1961; Applebury et al, 1970).

Both the non-covalent adsorption of two phosphate molecules on the enzyme at alkaline pH and the covalent phosphorylation at acidic pH are anticooperative processes. One site binds P_i strongly and its saturation occurs at

very low phosphate concentrations; the saturation of the second site is then much more difficult and much higher concentrations of P_i are necessary (Lazdunski, Petitclerc, Chappelet, and Lazdunski, 1969; Petitclerc, et al, 1970). This anticooperativity is also observed with AMP and is even higher than that observed with P_i (Lazdunski, et al, 1971). These observations have led the authors to propose a new kinetic mechanism for which the E. coli alkaline phosphatase is a model. This new mechanism, the Flip Flop mechanism, implies alternating functions for each of the two active sites of the phosphatase: the phosphorylation of one site is concurrent with the dephosphorylation of the other site.

Most of the microbial alkaline phosphatases studied so far have proven to be phosphate repressible. E. coli alkaline phosphatase is produced only when P_i becomes limiting to the growth medium (Horiuchi, Horiuchi, and Mizuno, 1959; Horiuchi, 1959; Torriani, 1960). Mutants of E. coli constitutive for alkaline phosphatase have been isolated (Torriani and Rothman, 1961).

The synthesis of alkaline phosphatase in E. coli K12 is controlled by three known genetic loci, one determining the structure of the enzyme and the other two regulating the amount of enzyme synthesized (Echols et al, 1961). Mutations within the structural gene lead to alterations in the primary structure of the protein. No differences in

fingerprints of the protein have been found to result from mutations in either of the regulatory genes (Echols et al, 1961).

Neurospora crassa possesses a repressible alkaline phosphatase (Kadner and Nyc, 1969) and also a non-repressible alkaline phosphatase (Kuo and Blumenthal, 1961), as well as a repressible acid phosphatase (Jacobs, Nyc and Brown, 1971). Aspergillus nidulans also produces two alkaline phosphatases, one repressible by inorganic phosphate and the other, non-repressible (Dorn, 1968). All the Staphylococcus aureus strains tested produced a repressible alkaline phosphatase (Shah and Blobel, 1967). However, the acid phosphatase produced by the same organism was not influenced by the different levels of P_i . The alkaline phosphatases of B. subtilis (Moses, 1967; Takeda and Tsugita, 1967), P. aeruginosa (Hou, Gronlund and Campbell, 1966), P. fluorescens (Friedberg and Avigad, 1967), Aerobacter aerogenes (Wolfenden and Spence, 1967), and Streptomyces fradiae (Majumdar and Majumdar, 1971) are also repressed by phosphate.

A wide variety of extracellular microbial diesterases have been described. These were reviewed as to their source, specificity, and mode of action in previous studies in this laboratory (Berry, 1965, 1969). The diesterase of M. sodonensis was shown to be exonucleolytic and to act on both native and denatured DNA as well as RNA and synthetic polynucleo-

tides such as poly A. 5'-nucleotidase activity was associated with the diesterase and could not be separated from it by any of the purification techniques employed. The final purified enzyme was homogeneous on gel filtration and ultracentrifugation. The two activities were shown to have identical pH optima and the same requirement for Mn^{2+} . The existence of a single protein was therefore postulated (Berry and Campbell, 1967a, b, 1970a).

Although an abundance of experimental data is available on the control of production of individual extracellular enzymes by microorganisms, few have studied the control of production of several such extracellular products by one organism. The best documented work was done on the spore-forming bacilli which release a multiplicity of biologically active substances, such as antibiotics, exoenzymes, toxins, into the culture medium (Schaeffer, 1969). In B. subtilis, fluctuations of levels of amylase, ribonuclease, and overall protease activities, for example in response to catabolite repression, have been shown to occur parallel to one another (Coleman, 1967). The ratio of the three enzymes to each other was maintained irrespective of whether maltose, starch, glycerol or glucose was included in the culture medium. Moreover, it was found that most of the sporulation-deficient mutants were deficient in the synthesis of several extracellular products and that mutant strains entirely lacking antibiotic, protease, lytic enzyme, or ribonuclease characters were all

pleiotropic sporulation-deficient mutants (Schaeffer, 1967). The various activities may not all be completely abolished in every mutant but as a rule they do not have the wild-type level. The pleiotropic mutants are single step, revertible mutants. Only the amylase-deficient mutants had all the other traits unchanged and sporulation-deficient mutants still produced amylase (Schaeffer, 1967).

The scarcity of information on the control of production of several extracellular enzymes by one organism prompted this study on the control of production of the four extracellular enzyme activities of M. sodonensis. In addition, the alkaline phosphatase of M. sodonensis was purified and characterized and the initial work on the 5'-nucleotidase was extended with a kinetic study of this enzyme.

MATERIALS AND METHODS

I. Organism and Growth Conditions

Micrococcus sodonensis ATCC 11880 was the organism used throughout these studies. Stock cultures were maintained on TCS agar. Cultures were grown aerobically at 30° unless otherwise specified.

II. Culture Media

The composition of the culture media used in these studies is given in Table I.

III. Control Studies

Cultures for growth or enzyme production control studies were obtained using 50 or 250 ml of media in 125 or 500 ml erlenmeyer flasks incubated with shaking at 30°. Five ml samples were removed at different time intervals from these cultures and centrifuged at 45,000 x g for 15 minutes, and the supernatant was assayed for enzyme activity.

IV. Enzyme Production

Cultures of M. sodonensis were grown in synthetic or TCS medium in a 10 liter capacity New Brunswick Microferm Laboratory Fermentor at 30° with agitation of 200 r.p.m. and

TABLE I

COMPOSITION OF CULTURE MEDIA USED FOR M. SODONENSIS

CONTROL STUDIES

<u>TCS Broth (Baltimore Biological Laboratories)</u>	gm/100ml
Trpticase Peptone (USP Pancreatic digest of casein)	1.7
Phytone peptone (USP Soy peptone)	0.3
Sodium chloride	0.5
Dipotassium phosphate	0.25
Dextrose	0.25

Modified TCS Broth

Trypticase peptone (USP Pancreatic digest of casein)	1.7
Phytone peptone (USP Soy peptone)	0.3
Sodium chloride	0.5
TRIS	0.484
Dextrose	0.25

Synthetic Medium

CaCl ₂	0.001
(NH ₄) ₂ MoO ₇ · 24H ₂ O	0.00008
ZnSO ₄	0.001
MnSO ₄ · H ₂ O	0.0008
H ₃ BO ₄	0.0004
CuSO ₄ · 5H ₂ O	0.0001
CoCl ₂ · 6H ₂ O	0.0001
FeSO ₄	0.0004
MgSO ₄ · 7H ₂ O	0.020
KCl	0.050
EDTA	0.00005
Sodium glycerol phosphate	0.010

TABLE I, continued

Biotin	0.000001
Lactic acid	0.50
TRIS	0.365
Glutamic acid	0.50
NH ₄ Cl	0.050

<u>Synthetic Medium+Neopeptone (Difco)</u>	
Neopeptone	1.0
Synthetic medium (as above)	100 ml

<u>Synthetic Medium+Casamino Acids (Difco)</u>	
Casamino acids	0.5 or 2.0
Synthetic medium	100 ml

<u>Synthetic Medium+P_i</u>	
P _i (Na ₂ HPO ₄)	0.0142
Synthetic medium	100 ml

aeration rate of 8 liters of air per minute. Cells were harvested at 24 hrs in late logarithmic phase in a Sharples centrifuge. The supernatant was concentrated and dialysed by ultrafiltration using an Amicon Model 400 cell and a Diaflo PM-10 membrane. Samples were filtered at 5° under nitrogen at 20 p.s.i.

V. Enzyme Purification

1) Ion Exchange Chromotography. Twenty liters of culture supernatant were concentrated to about 50 ml and dialysed against 0.02 M Tris-HCl, pH 7.6 before being applied to a DEAE-cellulose column, 63 cm by 3 cm, equilibrated at 5° with the same buffer. Then 500 ml of the equilibrating buffer were used to wash the column before eluting the protein with 1,200 ml NaCl gradient of 0 to 0.45 M NaCl in 0.02 M Tris-HCl, pH 7.6. Five ml fractions were collected and assayed for diesterase, alkaline phosphatase and 5'-nucleotidase activity.

2) Gel Filtration. The different enzyme fractions from the DEAE-cellulose eluate were concentrated to a volume of 4 to 6 ml, dialyzed and applied to a Sephadex G-200 column, 2.5 by 28 cm, with a pressure head of 10 cm. The Sephadex was previously equilibrated at 5° with 0.02 M Tris-HCl buffer, pH 8.8, and the enzyme was eluted with the same buffer. Four to five ml fractions were collected.

3) Ammonium Sulfate Fractionation. The Sephadex G-200 eluate was then concentrated to a volume of 5 to 15 ml and subjected to an ammonium sulfate fractionation. The ammonium sulfate was added slowly to the enzyme solution, stirred with a magnetic bar and kept at 0° in an ice-water bath. After 30 to 60 minutes, the 40% ammonium sulfate precipitate was centrifuged down at 45,000 x g. The supernatant ammonium sulfate concentration was raised from 40% to 50% and left overnight at 5° before being centrifuged at 45,000 x g. Most of the enzyme activities were found in this 40-50% ammonium sulfate cut. The precipitate was solubilized in 5 to 10 ml of 0.02 Tris-HCl, pH 8.8, and dialyzed against the same buffer.

4) Ultracentrifugation. Some enzyme preparations were further purified by placing 0.5 ml of enzyme on 4.5 ml of 20% sucrose buffered with 0.01 M Tris-HCl, pH 8.8, or on 4.5 ml of a linear gradient of 5 to 20% sucrose in the same buffer, and centrifuging for 23 hours at 50,000 rpm in a L2-65B Beckman ultracentrifuge, SW 50.1 rotor. Ten drop fractions were collected from the bottom of the tube and the 4 to 5 fractions containing 80 to 90% of the enzyme activity were pooled.

These fractions were dialyzed against 0.1 M phosphate buffer, pH 7.2, or 0.02 M Tris-HCl buffer, pH 8.8, and analyzed in a Spinco Model-E analytical ultracentrifuge. Sedimentation

velocity was measured with a rotor speed of 52,000 rev/min, bar angle 55° , rotor temperature 5° or 20° .

5) Disc Gel Electrophoresis. Polyacrylamide gel electrophoresis was carried out according to the method of Davis (1964). Crude extracellular protein was obtained from 18-22 hr cultures of M. sodonensis. After centrifugation at 45,000 x g for 20 minutes, the clear supernatant was dialyzed for 16 hrs against 0.01 M Tris-HCl buffer, pH 8.3, and concentrated with polyethylene glycol. 0.2 ml samples, in 20% sucrose, containing 100 to 150 μ g protein were applied on top of the spacer gel. In the case of the purified enzymes, 20 to 100 μ g of protein were incorporated into a sample gel. Two mA/tube were applied for around two hours. Protein was located by staining the gels with buffalo black or coomassie blue for 15 hrs and 2 hrs respectively, and destaining with 7% acetic acid in a Canalco 1801 Quick Gel Destainer or by prolonged exposure to 7% acetic acid.

Enzyme activity in the gels was located by slicing the gel in 2mm slices, extracting with 0.01 M Tris-HCl, pH 8.8, for 18 hours, and performing the appropriate assays. Activity was correlated with the protein bands by comparison with duplicate gels stained as previously described. Alkaline phosphatase activity was detected in gels by incubating the gels in a solution of phenolphthalein phosphate

("Phosphatrate Alkaline", Warner-Chilcott Lab. Inc., used as directed by the manufacturer i.e., one drop per ml of water).

VI. Preparation of Cell-Free Extracts

Cell-free extracts were prepared by harvesting cells in the late logarithmic phase, washing them twice with distilled water and resuspending them in 0.02 M Tris-HCl, pH 7.6, to give an absorbance at 600 nm of 20. One hundred μgm of lysozyme were added per ml of this cell suspension and incubated at 37° for 30 minutes and then centrifuged at $45,000 \times g$ for 20 minutes to remove debris. Such treatment was found to release 68% of the total cell protein (Shobe, 1970).

VII. Enzyme Assays

1) Alkaline Phosphatase. Enzyme activity was determined at 37° using PNPP (0.2 μmoles) as the substrate. The reaction mixture (1.0 ml) contained 50 μmoles glycine - NaOH buffer, pH 10.3, and 1.7 μmoles of CaCl_2 . The increase of absorbance at 405 nm was measured with a Gilford recording spectrophotometer. Enzyme activity was also determined using other substrates, as indicated in the text, at equal

concentration, in identical reaction mixtures. The reaction was stopped using 0.2 ml of 30% TCA and the amount of P_i released was determined by the Ames-Dubin colorimetric procedure. One unit of activity is the amount of enzyme which releases 1 μ mole of P_i (or 1 μ mole of p-nitrophenol) per minute.

2) 5'-Nucleotidase. Enzyme activity was determined at 37° using AMP (0.5 μ moles) as the substrate. The reaction mixture (1.0 ml) contained 33 μ moles Tris, 13 μ moles $MgCl_2$, 1.7 μ moles $CaCl_2$, 1.7 μ moles $MnCl_2$, 10 μ moles mercaptoethanol and 1 μ mole EDTA, at a final pH of 8.8. The reaction mixture and enzyme were incubated at 37° for 30 minutes before adding the substrate. The reaction was terminated by adding 0.2 ml of 30% TCA and P_i released was quantitated by the Ames-Dubin method.

When ^{14}C -AMP was used as substrate, the assay was scaled down to 0.1 ml assay volume. The reaction was terminated with the addition of 0.02 ml cold 4M formic acid and 0.1 ml was spotted on Whatman No 3MM paper with non-radioactive adenosine as marker. The developing solvent consisted of acetonitrile/0.1 M ammonium acetate (pH 7)/ammonium hydroxide (60:30:10) and was allowed to run 12 to 15 inches from the origin. The

adenosine spot was located with a short-wave ultraviolet lamp, cut out and placed in toluene scintillation fluid for counting in a Nuclear Chicago Mark I scintillation counter. The calculations required to express the results in terms of μ moles adenosine produced per minute and to determine the slopes and intercepts by the least squares method were done using an APL computer program (Smillie, 1969).

When 5'-nucleotidase activity was measured in the presence of contaminating alkaline phosphatase, CaCl_2 was omitted from the reaction mixture to prevent the latter enzyme from functioning.

3) Diesterase. The reaction mixture (0.1 ml) contained 3.3 μ moles Tris, 0.17 μ moles CaCl_2 , 1.3 μ moles MgCl_2 , 0.17 μ moles MnCl_2 , 1 μ mole mercaptoethanol, and 0.1 μ mole EDTA. The reaction mixture and the enzyme were incubated at 37° for 30 minutes before adding 0.2 mg RNA, as substrate. The reaction was stopped with 0.1 ml of cold 0.2% uranyl acetate in 10% TCA. Activity was followed by measuring the release of acid-soluble 260 nm absorbing material. One unit of activity is defined as the amount of enzyme which releases 1 μ mole of acid-soluble nucleotide per minute.

4) Transphosphorylase. Transphosphorylase activity was assayed by the method of Anderson and Nordlie (1967). A one ml reaction mixture contained 0.67 μ moles NADP^+ , 333 μ moles glucose, 10 μ moles PP_i or AMP, 1.7 μ moles CaCl_2 , 50 μ moles

Tris, pH 7.6, and 0.12 units of glucose-6-phosphate dehydrogenase. The absorbance at 340 nm at 37° was read with a Gilford recording spectrophotometer for five minutes before adding the M. sodonensis alkaline phosphatase to one of the two cuvettes. The A_{340} was monitored for an additional five minutes. Any glucose-6-phosphate produced in the transphosphorylase reaction will be oxidized by the glucose-6-phosphate dehydrogenase with the production of NADPH which absorbs at 340 nm.

5) Proteinase. The reaction mixture consisted of 5 mg soluble casein, 1.0 μmole Tris-HCl, pH 8.3, and 0.05 ml of culture supernatant in a 0.2 ml volume (37°). The reaction was terminated with 0.05 ml of 30% cold TCA and the acid-insoluble portion was removed by centrifugation after a fifteen minute incubation at 0°. The increase in acid-soluble 280 nm-absorbing material was followed. One unit is defined as that amount of activity causing an increase of 0.001 of A_{280} per minute in the acid-soluble fraction.

VIII. Isolation of Mutants. Mutants were isolated using the method of Omenn and Friedman (1970). Sixteen-hour cultures of M. sodonensis were centrifuged and the washed cells were suspended in phosphate buffer pH 7.0. Freshly-dissolved, membrane filter (Millipore Corp) - sterilized N-methyl-N'-nitro-N-nitrosoguanidine was added to give a final concentration of 100 μg/ml, and the suspensions were incubated at

37° for 30 minutes. Samples were diluted 50 times in nutrient broth and incubated overnight at 30°. The cells were diluted 10,000-fold and one or two drops were spread on deoxyribonucleic acid-acridine orange-agar plates. The plates were prepared by adding 40 µg of acridine orange to DNase Test Agar (Difco) before autoclaving. The resulting medium gave brilliant yellow-green fluorescence. Release and diffusion of diesterase from a colony led to a dark, non-fluorescent halo as the enzyme cleaved DNA to oligonucleotides and the agar served to quench free acridine orange. Fluorescence persisted around diesterase-deficient colonies. Presumptive diesterase-deficient colonies were inoculated into synthetic and TCS media and diesterase activity was assayed quantitatively at various times during the course of growth.

IX. Analytical Methods.

- 1) Protein was estimated by the technique of Lowry et al (1951).
- 2) P_i was determined by the method of Ames and Dubin (1960).
- 3) Total neutral sugar content was determined by the phenol-sulfuric acid technique of Dubois (Dubois et al, 1956). Glucose was employed as a standard.
- 4) Amino sugars were estimated by the Morgan-Elson technique as described by Ghuysen, Tipper and Strominger (1966). Glucosamine was employed as standard.

EXPERIMENTAL RESULTS

I. Multiplicity and Control of Extracellular Enzymes Produced by *M. sodonensis*

1. The Extracellular Enzymes of *M. sodonensis*. Four distinct enzyme activities have been demonstrated in the supernatant of logarithmic phase cultures of *M. sodonensis*: a) diesterase (at least two forms), b) 5'-nucleotidase, c) proteinase, and d) alkaline phosphatase. The initial separation of the several activities was effected by placing the 50 ml concentrate of the crude supernatant (6 liters) of a 24 hour culture of *M. sodonensis* grown on synthetic media and concentrated and dialyzed in the Diaflo, as described in Materials and Methods, on a DEAE-cellulose column. The protein was eluted as described in Materials and Methods and the 5 ml fractions were assayed for diesterase, 5'-nucleotidase and alkaline phosphatase activity. Figure 1 gives the elution profile obtained. The studies of each of these activities are discussed separately.

a) Diesterases. Contrary to the opinion expressed by Berry and Campbell (1967a, b) that there is a single diesterase produced by *M. sodonensis*, several techniques employed in this investigation revealed the presence of two diesterases. One could be freed from 5'nucleotidase contamination. Its peak

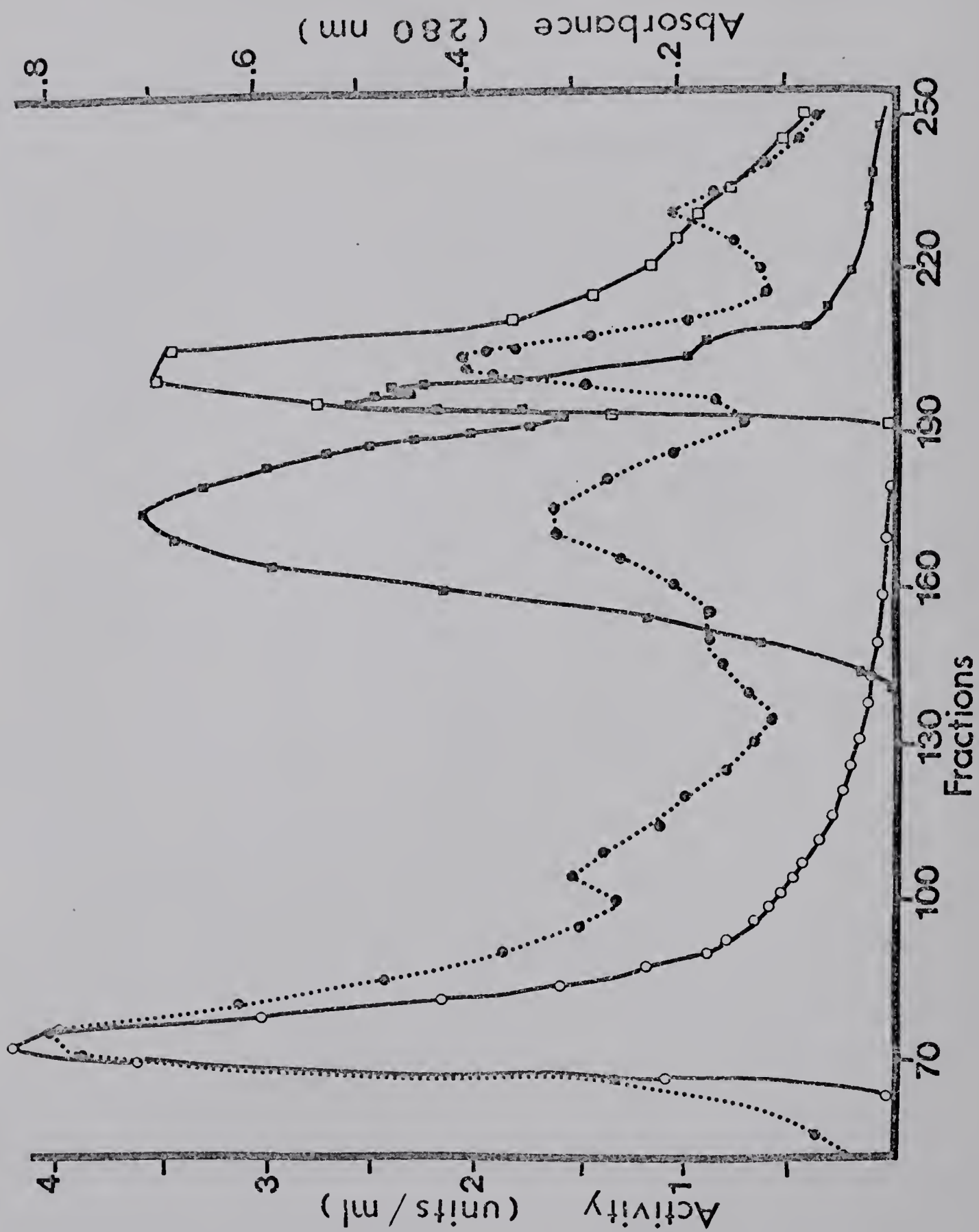
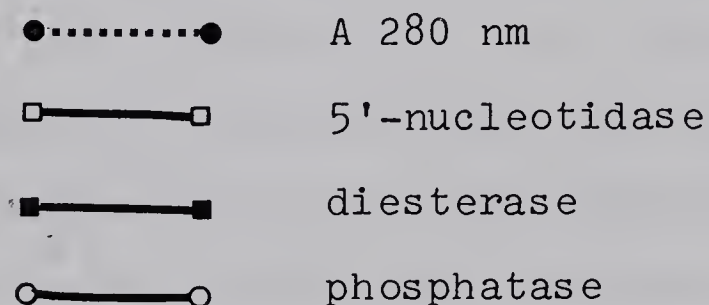


FIGURE 1

ELUTION PROFILE OF M. SODONENSIS EXTRACELLULAR ENZYMES FROM DEAE - CELLULOSE COLUMN. The column, 63 by 3 cm. was equilibrated with 0.02 M Tris-HCl buffer, pH 7.6 and the protein was eluted with 1200 ml NaCl gradient of 0 to 0.45 NaCl in 0.02 M Tris buffer, pH 7.6. Five ml fractions were collected.



occurs around fraction 175 in Figure 1. The other could not be freed from 5'-nucleotidase contamination and its peak occurs around fraction 195 in Figure 1.

Two forms of diesterase were also separable using a BioGel HTP-hydroxyapatite column, 7.5 cm by 1.5 cm, equilibrated with 0.001 M phosphate buffer, pH 7.7. Concentrated fractions, from a Sephadex G-200 column, with both 5'-nucleotidase and diesterase activities, were applied to the column and washed with 40 ml of 0.001 M phosphate buffer. About one fifth of the diesterase activity was eluted with the wash, but none of the 5'-nucleotidase activity. The rest of the enzyme activities were eluted as a single peak at a concentration of phosphate of approximately 0.0075 M.

The presence of at least two forms of diesterase was also evident when concentrated fractions from a Sephadex G-200 column, with both 5'-nucleotidase and diesterase activities, were subjected to gel electrophoresis. When the gels were cut into sections, two or three peaks of diesterase activity were found. The proteins ran toward the anode (+) and the leading protein band was only 1 mm behind the riboflavin band. One of the protein bands corresponding to diesterase activity is very close to or indistinguishable from the leading protein band corresponding to the 5'-nucleotidase activity as shown in gel 4 in Figure 2. In only a few cases was it possible to obtain sections with 5'-nucleotidase activity without contaminating diesterase

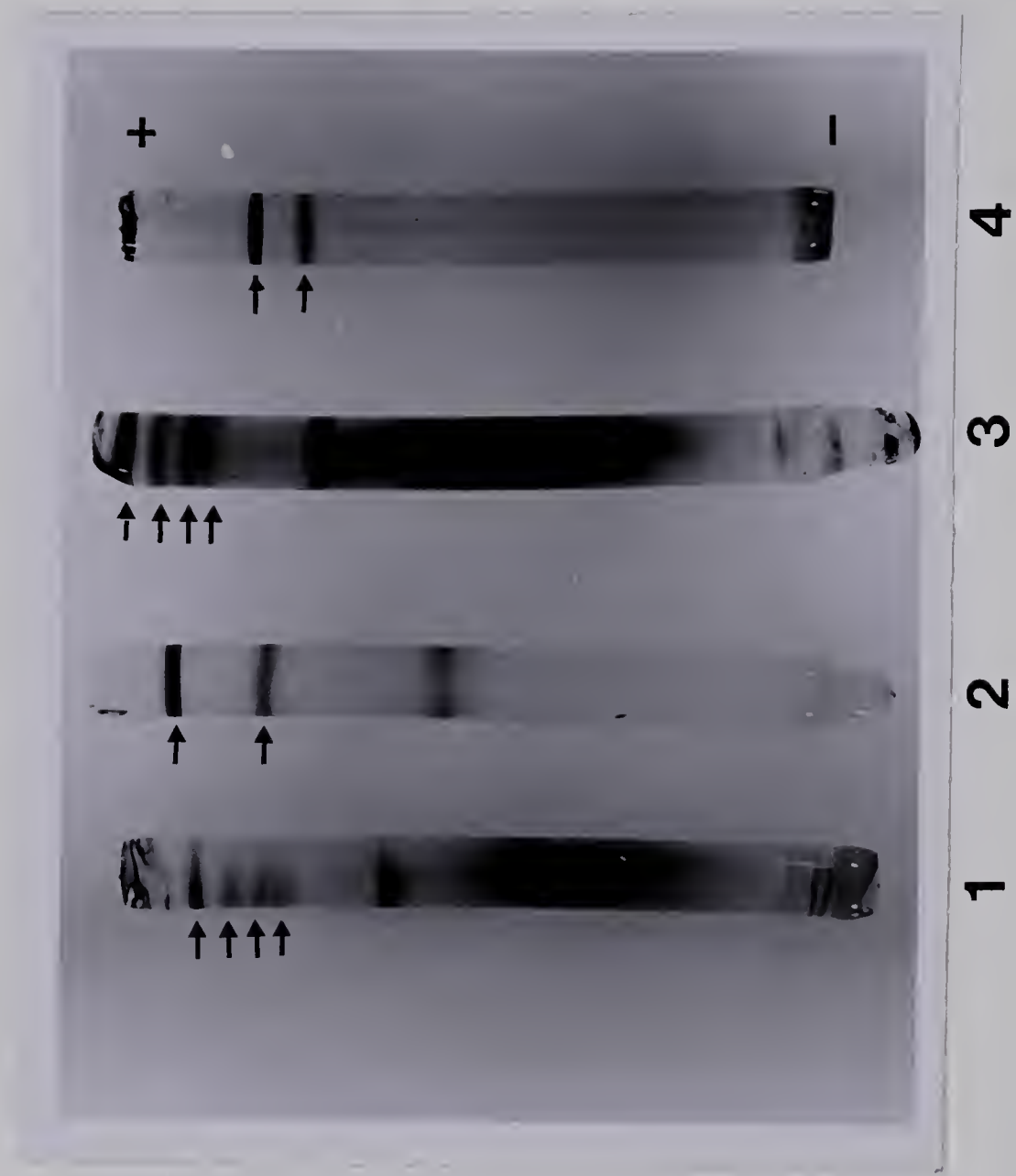


FIGURE 2

POLYACRYLAMIDE GEL ELECTROPHORESIS OF VARIOUS PREPARATIONS OF M. SODONENSIS DIESTERASE. The band indicated by the first arrow at the top has both 5'-nucleotidase and diesterase activities. The other bands have only diesterase activity.

- 1, 3 - gel electrophoresis of crude supernatants with multiple diesterase bands
- 2 - gel electrophoresis of crude supernatant with two diesterase bands
- 4 - gel electrophoresis of purified 5'-nucleotidase and diesterase

activity. The purified enzyme preparations with both 5'-nucleotidase and diesterase activities gave one or more additional bands of diesterase activity on gels. Crude extracellular protein subjected to gel electrophoresis also gave a variable number of bands corresponding to diesterase activity (Figure 2). The variable number of diesterase bands could be due to the action of the protease on the diesterase. However, M. sodonensis cultures consistently showed the production of at least 2 diesterases under these conditions of growth.

b) 5'-Nucleotidase. The 5'-nucleotidase eluted from the DEAE-cellulose column as a single peak at a NaCl concentration of approximately 0.35 M. A small amount of diesterase was present in most of the fractions (Figure 1). When fractions 189-220 (Figure 1) were pooled, concentrated and rechromatographed on a DEAE column equilibrated with 0.25 M NaCl and eluted with a 1400 ml gradient of 0.25 to 0.45 M NaCl in 0.02 M Tris-HCl buffer, pH 7.6, some further separation of diesterase from the 5'-nucleotidase was effected but the diesterase still trailed into the 5'-nucleotidase area (Figure 3). Using similar enzyme preparations from different enzyme purification runs, the same experiment was repeated using AMP (2mM) or adenosine (5 mM) in the eluting gradient or substituting TEAE-cellulose for DEAE-cellulose, all other experimental conditions, such as size of column, eluting gradient, etc., remaining constant. The elution

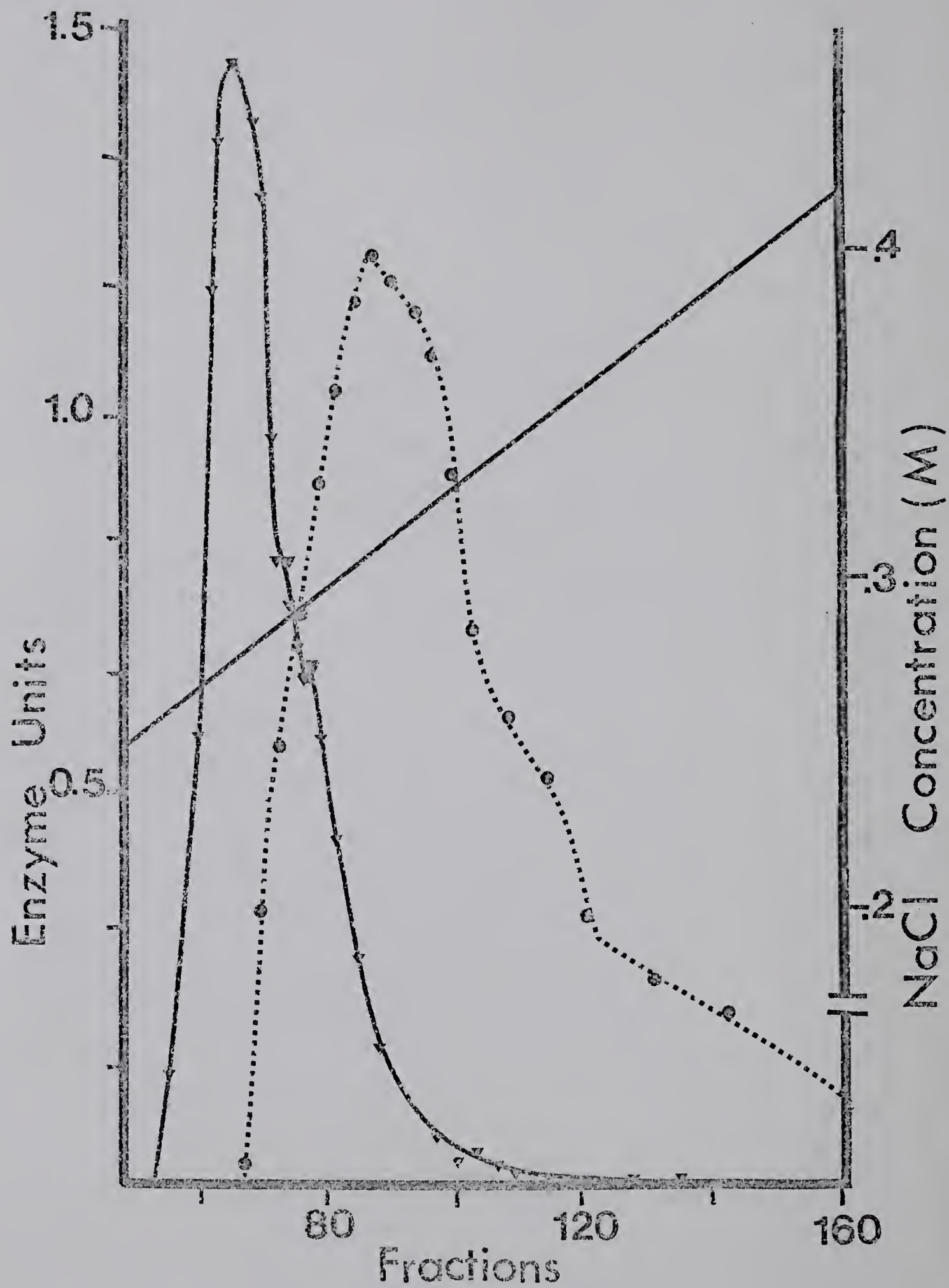
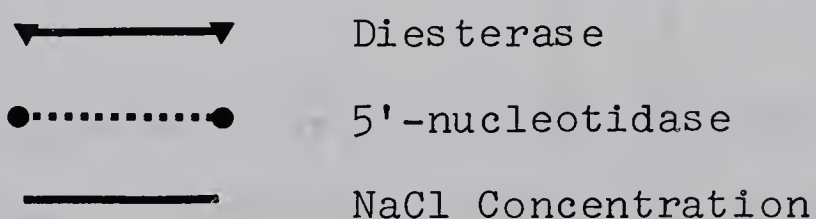


FIGURE 3

RECHROMATOGRAPHY ON DEAE-CELLULOSE OF M. SODONENSIS 5'-NUCLEOTIDASE. Fractions 189-220 from a DEAE cellulose column (Figure 1) were applied to a second DEAE column equilibrated with 0.25 M NaCl and eluted with a 1400 ml gradient of 0.25 to 0.45 M NaCl in 0.02 M Tris-HCl buffer, pH 7.6. Five ml fractions were collected.



profile was not altered under any of these conditions. When fractions from the DEAE column containing both diesterase and 5'-nucleotidase activity were concentrated and applied to a Sephadex G-200 column or a Sepharose 6B column, 40 cm by 2.5 cm, and eluted with 0.02 M Tris-HCl, pH 7.6, a single peak of enzyme activity was obtained. Also the Schlieren pattern from the analytical ultracentrifugation (performed as indicated in Materials and Methods) gave a single peak as previously observed by Berry and Campbell (1967a).

A kinetic study of the 5'-nucleotidase activity is discussed in a separate section. The diesterases and the 5'-nucleotidase were not further characterized. The exact nature of the relationship between the 3 is not clear although it would appear that 3 distinct enzymes are present.

c) Proteinase. The extracellular proteinase activity was demonstrated using crude culture supernatants of 18 to 24 hour cultures grown on synthetic and TCS media. Both media gave around 3 units of proteinase per mg dry weight of cells (Table III) when assayed as indicated in Materials and Methods using 5 mg soluble casein as substrate. When an equal amount of bovine serum albumin was used as substrate three times less acid-soluble material absorbing at 280 nm was released.

When 10^{-3} M EDTA, 13×10^{-3} M MgCl_2 , 1.7×10^{-3} M CaCl_2 , or 1.7×10^{-3} M MnCl_2 were incorporated into the reaction mixture given in Materials and Methods, the proteolytic activity was not affected, indicating that the proteinase is probably not a metalloenzyme. A pH curve done with overlapping buffers at 0.05 M concentrations (see Figure 13 for buffers used for the various ranges) indicated that the pH optimum is in the high alkaline range (pH 10-11). Incubation of the enzyme with 10^{-3} M diisopropyl fluorophosphate for 30 minutes at 37° resulted in total inactivation of the enzyme. The alkaline pH optimum, the insensitivity of the enzyme to EDTA, and the inactivation by diisopropyl fluorophosphate all suggest that the enzyme fits into the category of the alkaline proteinases produced by several other microorganisms (Davies, 1963). No further purification or characterization was attempted.

d) Alkaline Phosphatase. This enzyme is discussed in a separate section.

2. Extracellular Nature of the Enzymes. To ascertain the normal site of activity of the enzymes, 50 ml of synthetic and TCS media in 125 ml erlenmeyer flasks were inoculated with a standard washed inoculum of 18 hour cells of M. sodonensis. At designated intervals, 5 ml aliquots were removed, centrifuged at $45,000 \times g$ for 20 minutes, and the clear supernatant analyzed for the presence of phosphatase (PNPP assay), diesterase (RNA assay), 5'-nucleotidase (AMP

assay) and proteinase (casein assay). Details of the assay procedures are given in Materials and Methods. The results are given in Figures 4 and 5. It can be seen that the four enzyme activities appeared in the culture supernatant during logarithmic growth and fell or levelled off when the stationary phase was reached, indicating that the release of the enzymes is not due to lysis or leakiness of the cells as might occur in older cultures.

The cells centrifuged down in the above experiment were washed with 0.02 M Tris-HCl, pH 8.8, and resuspended in the same buffer. Enzyme assays carried out with the washed cells indicated that less than 5% of any of the enzyme activities was associated with the washed cells at any stage of growth of M. sodonensis. At the time intervals, 16, 20, 23 hours, cell-free extracts of the cells recovered from the two media were prepared using the lysozyme technique. Analyses of these revealed a maximum of 0.1 - 0.2% of the total activity of the four enzymes was associated with the cell-free extracts. The cell-free extract itself did not inhibit enzyme activity.

The question was asked whether the enzymes were periplasmic but released into the culture medium due to the presence of some component of the medium. Tris is known to cause the release of periplasmic enzymes in certain microorganisms, so it was replaced in the synthetic medium by the same concentration of Hepes. No change in the amount or

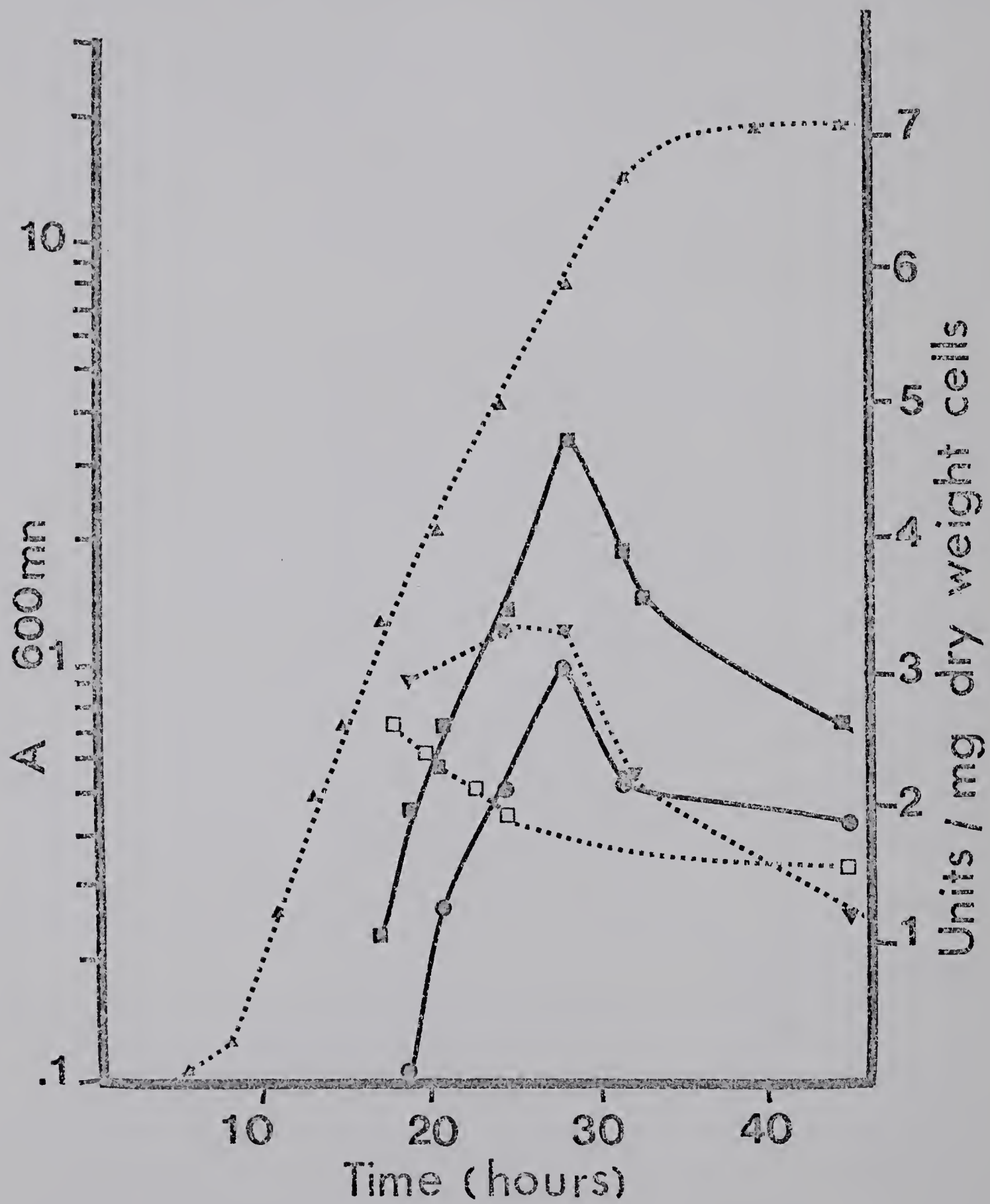


FIGURE 4

GROWTH RESPONSE AND ENZYME PRODUCTION BY

M. SODONENSIS IN SYNTHETIC MEDIUM

▲.....▲	Growth (A 600 nm)
■————■	Alkaline phosphatase (units/mg dry weight cells x 10 ²)
▼.....▼	5'-Nucleotidase (units/mg dry weight cells x 10 ²)
●————●	Diesterase (units/mg dry weight cells x 10 ²)
□.....□	Proteinase (units/mg dry weight cells)

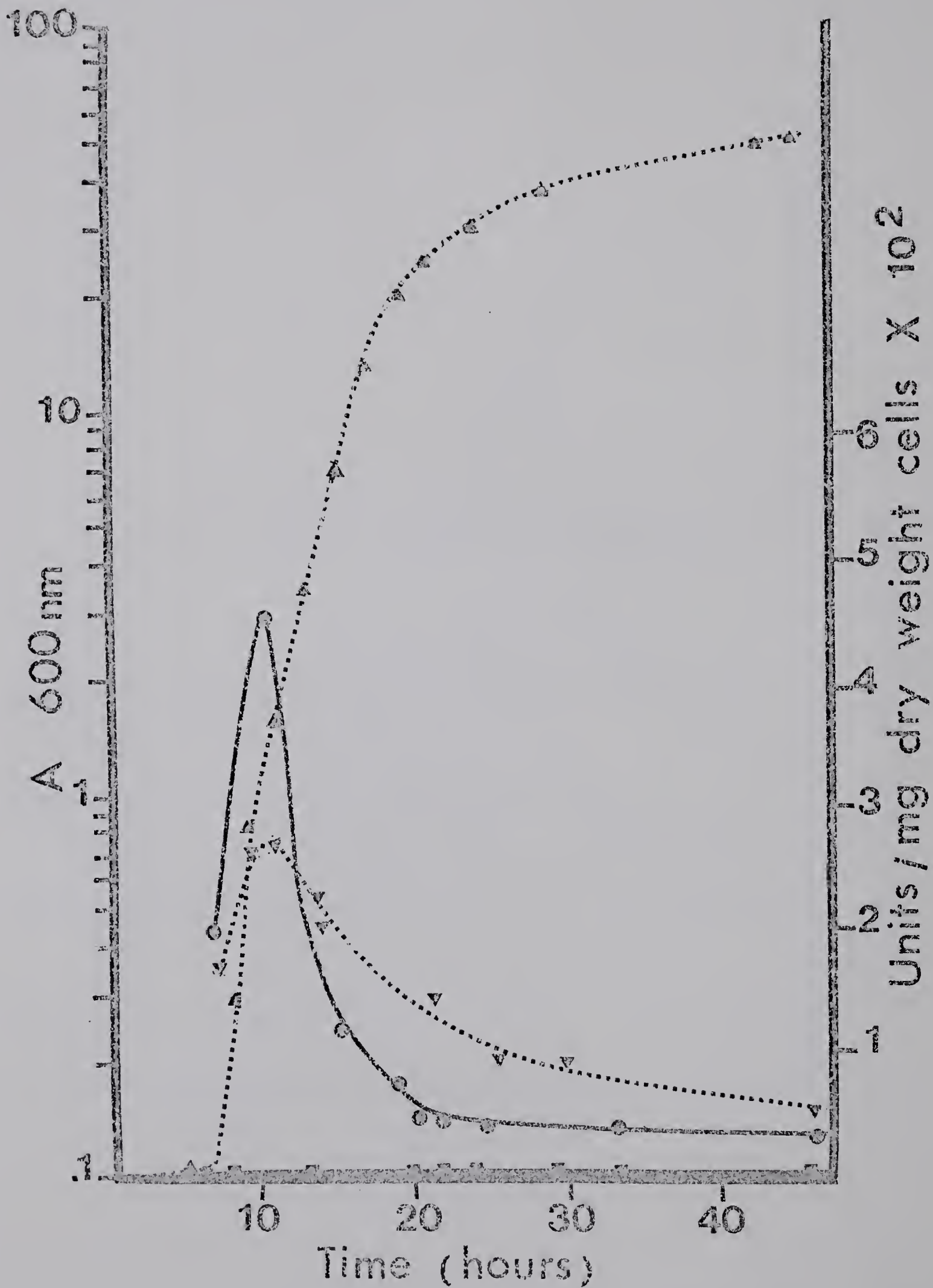
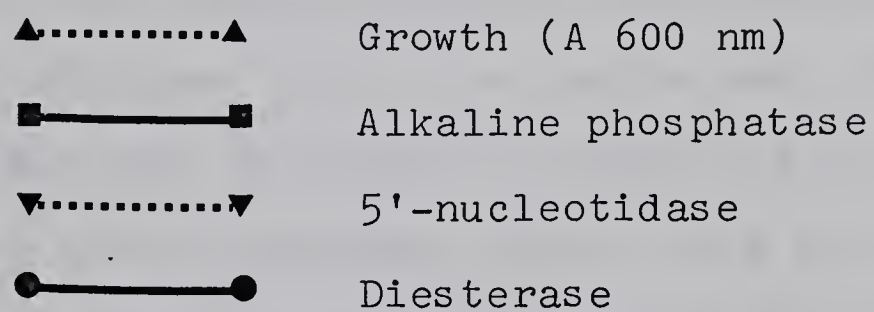


FIGURE 5

GROWTH RESPONSE AND ENZYME PRODUCTION BY

M. SODONENSIS IN TCS BROTH

distribution of enzymes released into the supernatant or associated with the cells was noted. EDTA, present in the synthetic medium, is also known to cause the release of periplasmic enzymes. Since its concentration is only 10^{-6} M, it is doubtful if it would have an effect. However, to confirm this, E. coli C₄F₁ was grown in the synthetic medium and observed to release none of its periplasmic alkaline phosphatase into the medium.

Thus it is concluded that all four of the enzymes are true extracellular enzymes.

3. Effect of Growth Medium on Production of Extracellular Enzymes. For comparison, enzyme production and growth response were monitored with time in TCS and synthetic media cultures (Figures 4, 5). As can be seen, the amounts of 5'-nucleotidase and diesterase present per mg dry weight of cells in the late logarithmic phase are 3 to 4 times higher in the synthetic medium than in the TCS medium and no alkaline phosphatase is produced in the TCS medium. To find out what components in the TCS broth are responsible for this repression, M. sodonensis was grown in the media listed in Table I. Fifty ml of the different media in 125 ml erlenmeyer flasks were inoculated with a standard washed inoculum of 18 hour cells of M. sodonensis. At designated intervals, 5 ml aliquots were removed, centrifuged at 45,000 x g for 20 minutes, and the clear supernatant analyzed for

the presence of the extracellular enzymes. In recording the number of enzyme units produced per mg dry weight cells, the highest value obtained in the late logarithmic phase was used.

TCS broth has a P_i concentration of 1.7×10^{-2} M, whereas the synthetic medium contains 3.2×10^{-4} M P_i . To lower the phosphate concentration in the TCS broth, a modified broth was prepared using Tris instead of phosphate buffer. The modified TCS medium still had a P_i concentration of 2.6×10^{-3} M. To test the effects of P_i in synthetic medium, P_i was added to give a final concentration of 1.3×10^{-3} M. The effect of added organic nitrogen to the synthetic medium was studied using a 1% addition of Neopeptone (Difco) to the synthetic medium. This addition did not significantly alter the P_i concentration of the medium.

Table II gives the enzyme concentrations produced in each of these media. The nucleotidase, phosphatase and diesterase activities are all present in the synthetic medium and the addition of P_i to this medium results in the repression of all three enzymes. The addition of Neopeptone to the synthetic medium results in the production of over 50% more cells and of more nucleotidase, diesterase and phosphatase per gm dry weight of cells.

Although nucleotidase and diesterase activities are produced in the TCS broth, no phosphatase is produced in this medium (Figure 4; Table II). The amount of cells produced is three times that found in the synthetic medium but ap-

TABLE II. EFFECT OF ALTERATION OF GROWTH MEDIA ON EXTRACELLULAR
ENZYMES PRODUCTION BY M. SODONENSIS

Units/gm dry wt. cells					
Medium	P _i (M)	Nucleotidase	Diesterase	Phosphatase A600nm	
TCS	1.7x10 ⁻²	9.0	3.9	0	40
Modified TCS	2.6x10 ⁻³	10.0	4.4	0	36
Synthetic	3.2x10 ⁻⁴	28.8	17.9	23.7	14
Synthetic + P _i	1.3x10 ⁻³	0.5	1.7	0	18
Synthetic + Neopeptone	3.4x10 ⁻⁴	49.4	24.3	27.9	32
Synthetic + Neopeptone + P _i	3.4x10 ⁻³	24.0	11.5	0.5	34
Synthetic + Casamino acids	3.3x10 ⁻³	39.6	14.8	0	30
Mutant					
TCS		7.3	0.8	0	43
Synthetic		24.0	0	18.5	14
Synthetic + P _i		0.3	0	0	18
Synthetic + Neopeptone		24.7	0.8	24.6	32

proximately the same amount of nucleotidase and diesterase are produced, resulting in about one third the amount of enzyme per gram dry weight of cells in TCS medium as compared to the synthetic medium. The modified TCS broth gave the same results as the TCS broth with the phosphate buffer.

The amount of P_i present in TCS medium is ten times that present in synthetic medium with added phosphate and yet the repression of diesterase and 5'-nucleotidase production by phosphate is much more marked in the synthetic medium. Consequently, P_i concentration alone is not the only factor involved; other factors in the TCS modify the effect of P_i on enzyme production. This was verified by adding P_i as well as Neopeptone to the synthetic medium. The Neopeptone reversed the effect of P_i about 50% in the case of the 5'-nucleotidase and the diesterase (Table II). The alkaline phosphatase, on the other hand, is effectively repressed in all cases indicating that the control of alkaline phosphatase production by P_i is independent of or more stringent than the effect of P_i on the diesterase and the 5'-nucleotidase.

The addition of 2% casamino acids to the defined medium did not markedly affect the production of 5'-nucleotidase or diesterase but completely repressed the production of the alkaline phosphatase (Table II). The individual amino acids, in concentration equal to or in excess of those present in

the casamino acids, did not repress alkaline phosphatase production, suggesting that the repression is due to the presence of more than one amino acid.

Gel electrophoresis of concentrated culture supernatants from the different media indicated that the presence or absence of P_i in either the TCS medium or the synthetic medium did not alter qualitatively the protein bands appearing on the gels. One band corresponding to diesterase activity, however, was always fainter in the case of cultures grown in the presence of P_i (Figure 6). This suggests that inactive proteins corresponding to the enzymes may still be produced under conditions of P_i "repression".

4) Control of Enzyme Production by a Mutant Strain.

Mutants of M. sodonensis were produced and screened for as indicated in Materials and Methods. A mutant with altered diesterase production and a growth rate comparable to that of the wild type organism was selected for further study and designated as isolate 2-B. This mutant failed to produce any diesterase when the cells were grown in synthetic medium (Table II). The 5'-nucleotidase and the alkaline phosphatase were produced in normal amounts and seemed to be unaffected by the mutation. When the synthetic medium was supplemented with P_i to give a final phosphate concentration of 1.32×10^{-3} M, none of the enzymes were produced by the wild type. The mutation seems to involve the

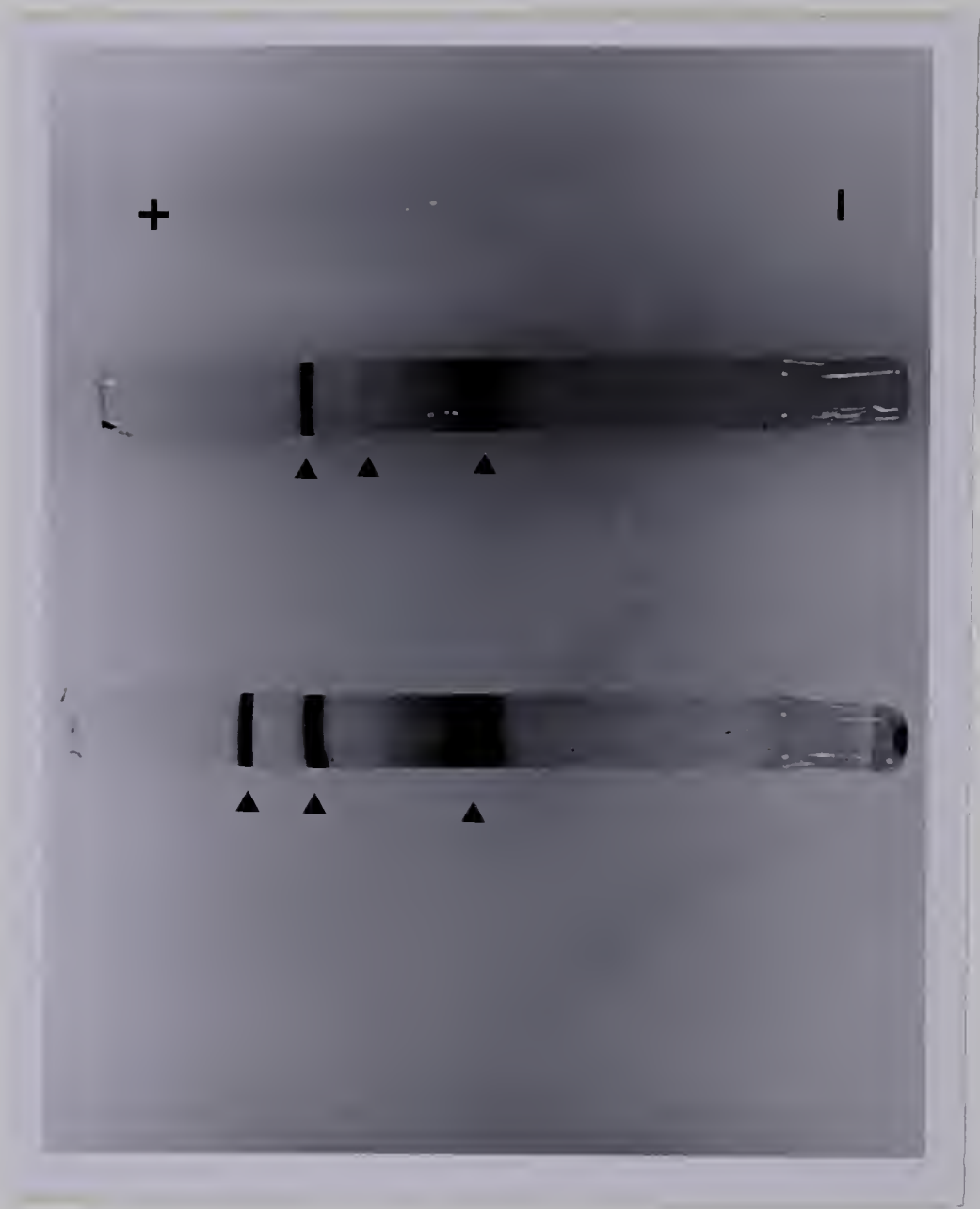


FIGURE 6

POLYACRYLAMIDE GEL ELECTROPHORESIS OF CULTURE SUPERNATANTS
OF M. SODONENSIS GROWN IN SYNTHETIC MEDIUM WITH AND WITHOUT
ADDED P_i

- 1 - Gel electrophoresis of supernatant from synthetic medium culture. The top band possesses both 5'-nucleotidase and diesterase activities. The middle band has only diesterase activity. The bottom band corresponds to alkaline phosphatase activity.
- 2 - Gel electrophoresis of supernatant from synthetic medium culture in which the final P_i concentration was 1.32×10^{-3} M. The three bands indicated correspond to those found in gel 1, but no enzyme activity was detected in any of them.

loss of or the reduced production of a protein, since gel electrophoresis of concentrated supernatants of 2-B cultures revealed no new protein band but only the reduction of one of the protein bands corresponding to diesterase activity in the wild type (Figure 7). The disc gels obtained with the cultures of the mutant were indistinguishable from those obtained with supernatants of the wild type cultures grown in the presence of P_i .

5) Control of Proteinase Production. Culture supernatants of M. sodonensis, both mutant and wild type, grown in TCS and synthetic media, were assayed for proteinase activity and were found to possess approximately the same number of enzyme units per milligram dry weight of cells (Table III). The addition of P_i (final concentration 2.32×10^{-3} M) to the synthetic medium did not affect proteinase production. Substances known to alter proteinase production such as casamino acids (0.5 and 2.0%), Neopeptone (1%), Ca^{2+} (10^{-3} M), did not result in any significant change in the number of units of proteinase produced per milligram dry weight of cells (Table III). Furthermore, no difference between the mutant and the wild type was detected.

6) Production of Extracellular Carbohydrate. High molecular weight carbohydrate is released into the culture medium by M. sodonensis cells when grown in the synthetic medium. The dialyzed culture supernatant contains about



FIGURE 7

POLYACRYLAMIDE GEL ELECTROPHORESIS OF CULTURE SUPERNATANTS
OF WILD TYPE AND MUTANT STRAIN 2-B OF M. SODONENSIS GROWN
IN SYNTHETIC MEDIUM

- 1, 3 - gel electrophoresis of culture supernatant of wild type M. sodonensis, grown on synthetic medium. The top band indicated possesses both 5'-nucleotidase and diesterase activities. The next band has only diesterase activity.
- 2, 4 - gel electrophoresis of culture supernatant of mutant strain of M. sodonensis, grown on synthetic medium. The bands corresponding to those indicated in gels 1 and 3 are marked, but no diesterase activity occurs in either.

TABLE III

EFFECT OF ALTERATION OF GROWTH MEDIA ON PROTEINASE
PRODUCTION BY M. SODONENSIS

Media	Organism	Proteinase units/mg dry weight cells	A600nm
Synthetic	Wild type	3.1	14
Synthetic	2-B	3.0	13
Synthetic + P _i	Wild type	3.2	18
Synthetic + P _i	2-B	3.5	15
TCS	Wild type	2.9	38
TCS	2-B	3.1	36
Synthetic + 0.5% casamino acids	Wild type	3.5	23
Synthetic + Neopeptone	Wild type	3.1	27
Synthetic + Ca ²⁺	Wild type	3.4	16
Synthetic + 2% casa- mino acids	Wild type	3.6	28
Synthetic + glycine (-glutamate)	Wild type	3.5	9

10^{-2} M total neutral sugar as determined by the phenol-sulfuric acid technique of Dubois, using glucose as standard, and 10^{-4} M hexosamine as determined by the Morgan-Elson technique using glucosamine as standard. About 90% of this non-dialyzable carbohydrate is separated from the alkaline phosphatase during the course of its purification on DEAE-cellulose (Table VI). It appears in the DEAE eluate as a broad peak extending from fraction 70 to fraction 200 (Figure 1). About 50% of that associated with the alkaline phosphatase after the DEAE step appears in the void volume of the Sephadex G-200 column used in the purification of the alkaline phosphatase (Figure 9), and about 80% of the carbohydrate still associated with the alkaline phosphatase after the gel filtration step remains in the 100% saturation supernatant in the ammonium sulfate fractionation of the enzyme (Table V). When sedimentation velocity analyses were carried out as indicated in Materials and Methods, the carbohydrate still associated with the alkaline phosphatase sedimented so slowly that it usually did not leave the meniscus during the duration of the run (2-3 hours). However, its exclusion from the Sephadex G-200 column indicated that its particle size or shape is different from that of the alkaline phosphatase.

To determine whether this carbohydrate is simply an excretion product of old or lysing cells, a growth curve of M. sodonensis cultured in the synthetic medium was done in .

correlation with the determination of the levels of hexosamine found in the supernatant at the various times. Figure 8 demonstrates that the rise in hexosamine production parallels the growth of M. sodonensis. Thus the carbohydrate is released at the same time as the extracellular enzymes.

A certain amount of protein remains associated with the carbohydrate fraction, suggesting that the carbohydrate fraction may contain glycoprotein. The amino sugar linkages of some amino acids are ~~alkali~~-labile. Carubelli, Bhavanandan and Gottschalk (1965) followed the release of N-acetyl-galactosamine from serine in ovine submaxillary gland protein by measuring the increase in absorption at 241 nm. The sugar was released by β -elimination and the α -aminoacrylic acid derivative of serine, which was formed concurrently, absorbs strongly at this wavelength.

Two carbohydrate fractions, totally devoid of enzyme activity, were saved in the course of the purification of the alkaline phosphatase (Table VI). One consisted of carbohydrate eluted in the void volume of the Sephadex G-200 column and the other of carbohydrate present in the supernatant saturated with ammonium sulfate (100%). These two carbohydrate fractions were suspended in 0.5 N NaOH for one hour and the increase at 241 nm was measured (Table IV). Assuming a molar extinction coefficient of 5300 for the aminoacrylic acid derivative, one μ mole of the derivative was formed per mg of protein per hour, with both carbohydrate

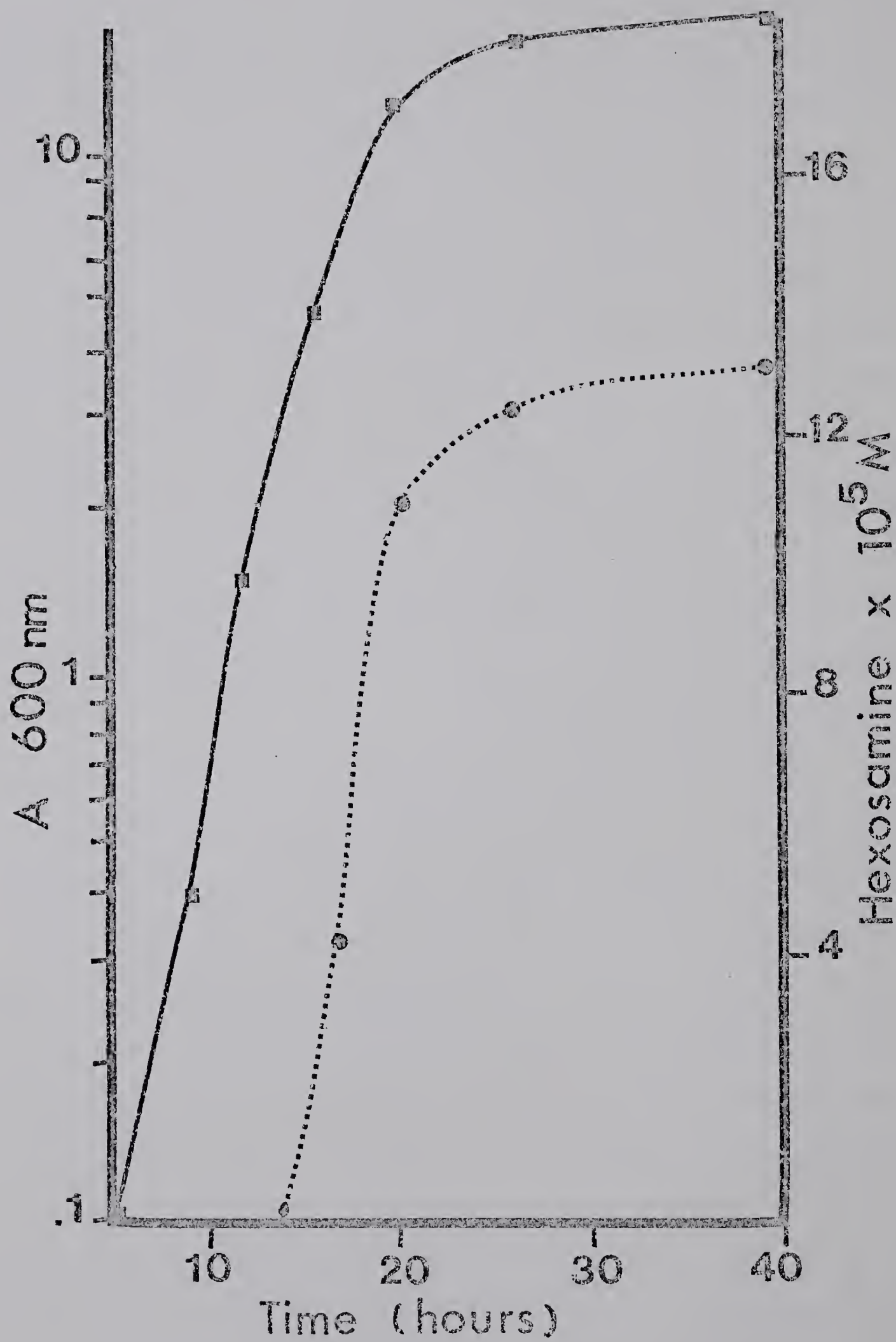


FIGURE 8

GROWTH RESPONSE AND HEXOSAMINE PRODUCTION BY

M. SODONENSIS IN SYNTHETIC MEDIUM

Hexosamine was detected by the Morgan-Elson technique using glucosamine as standard.

■————■ A 600 nm
●.....● hexosamine

TABLE IV. ALKALINE HYDROLYSIS OF EXTRACELLULAR
CARBOHYDRATE FRACTION OF M. SODONENSIS

Carbohydrate fraction	μ moles carbohydrate ¹ per ml	mg protein ² per ml	A 241 nm per mg protein	μ moles product per mg protein
Carbohydrate eluted in void volume of Sephadex G-200 column	6.4	0.3	5.46	1.03
Carbohydrate in supernatant of saturated $(\text{NH}_4)_2\text{SO}_4$ solution	14.1	0.28	5.53	1.04

¹detected by Dubois technique

²detected by Lowry technique

fractions. These data suggest that the carbohydrate fraction contains protein-carbohydrate linkages and therefore that the carbohydrate fraction contains glycoprotein.

II. THE ALKALINE PHOSPHATASE OF M. SODONENSIS

1. Purification

The concentrated culture supernatant from the Diaflo was applied to a DEAE-cellulose column, 63 cm by 3 cm, equilibrated with 0.02 Tris-HCl buffer, pH 7.6. Then, 500 ml of the equilibrating buffer were used to wash the column before eluting the protein with 1,200 ml NaCl gradient of 0 to 0.45 M NaCl in 0.02 M Tris buffer pH 7.6. Five ml fractions were collected and assayed for diesterase, alkaline phosphatase and 5'-nucleotidase activity as previously described. Figure 1 gives a typical elution profile. The alkaline phosphatase emerged as a single peak of activity at approximately 0.075 M NaCl, whereas the diesterase and the 5'-nucleotidase were eluted between 0.25 to 0.40 M NaCl. High molecular weight carbohydrate was eluted with a broad peak occurring between the alkaline phosphatase and the diesterase peaks. In subsequent purification runs, an NaCl gradient of 0.05 to 0.15 M NaCl was used to obtain a better separation of the carbohydrate and the alkaline phosphatase.

The pooled DEAE-cellulose eluate was then put on a Sephadex G-200 column, 2.5 by 28 cm, with a pressure head of 10 cm. The Sephadex was previously equilibrated with 0.02 M Tris-HCl buffer pH 8.8, and the enzyme was eluted with the

same buffer. Some of the high molecular weight carbohydrate appeared in the void volume and trailed into the enzyme region (Figure 9). Four and one half ml fractions were collected and fractions 29-40 were pooled.

The G-200 Sephadex eluate was then concentrated and subjected to an ammonium sulfate fractionation as described in Materials and Methods. As shown in Table V, 78% of the enzyme activity recovered was precipitated between 40-50% saturation with ammonium sulfate. Although about one half of the total enzyme activity was always lost in this step, removal of most of the carbohydrate was achieved.

Table VI gives typical purification results, with a 20% recovery of the enzyme and a 33-fold purification. The Schlieren pattern of the enzyme in the analytical ultracentrifuge at this stage of purification gives a slower sedimenting peak of carbohydrate as well as the protein peak. The remaining carbohydrate was removed by placing 0.5 ml of enzyme on 4.5 ml of 20% sucrose buffered with 0.01 M Tris-HCl, pH 8.8, and centrifuging for 23 hours at 50,000 rpm on a L2-65B Beckman ultracentrifuge, SW 50.1 rotor. The fractions containing the enzyme were dialyzed against 0.1 M phosphate buffer, pH 7.2, and analyzed in a Spinco Model-E analytical ultracentrifuge. Sedimentation velocity was measured with a rotor speed of 52,000 rev/min, bar angle 55° , rotor temperature 5° . Figure 10 indicates that the enzyme now gives a single homogeneous peak. The $s_{20,w}^{\circ}$ was 4.67.

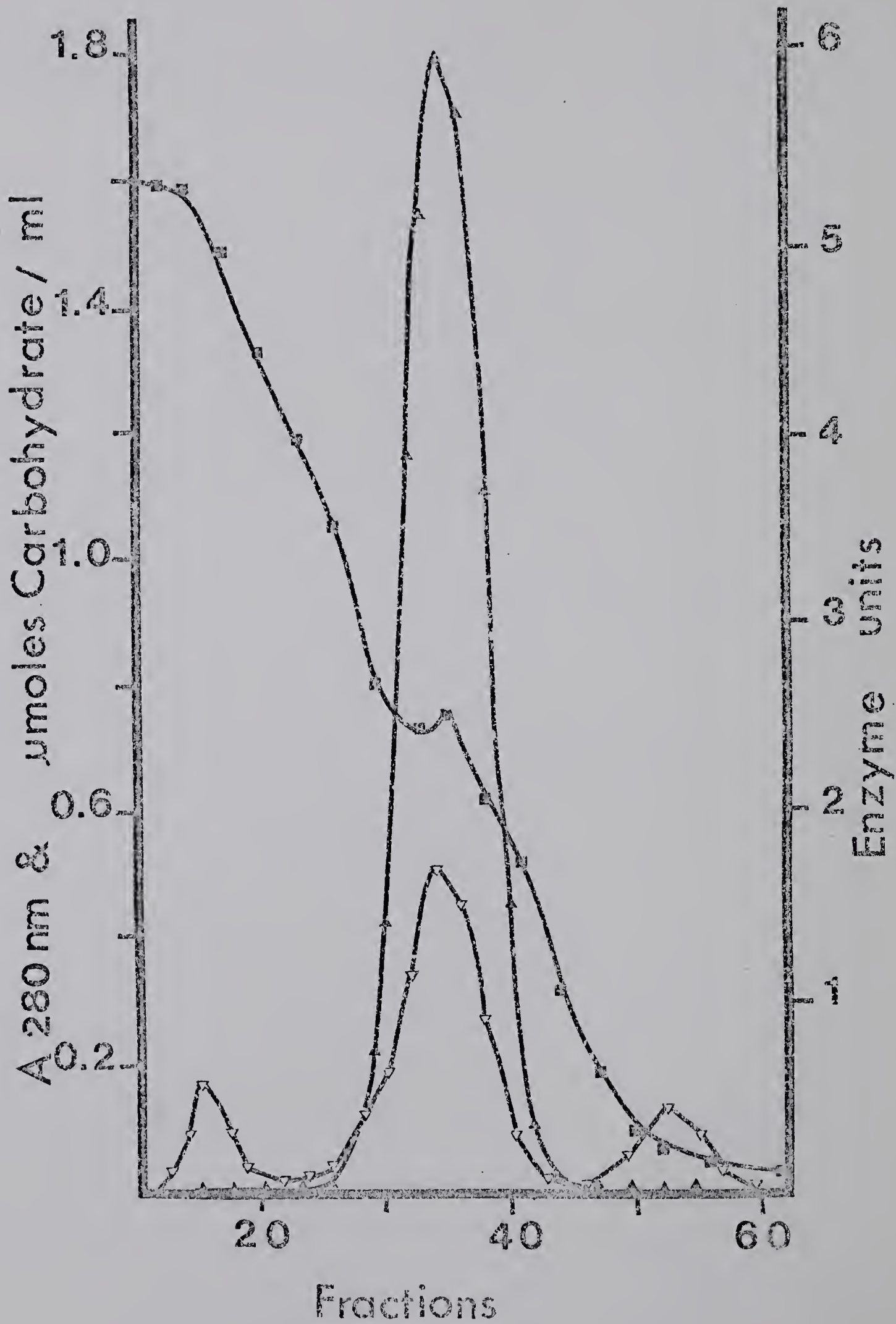


FIGURE 9

ELUTION PROFILE OF M. SODONENSIS ALKALINE PHOSPHATASE
FROM SEPHADEX G-200

Fractions 65 to 85 from the DEAE-cellulose column (Figure 1) were pooled, dialyzed and concentrated to 5 ml and applied to a Sephadex G-200 column, 2.5 by 28 cm and eluted with 0.02 M Tris-HCl buffer, pH 8.8. Four and one half ml fractions were collected and fractions 29-40 were pooled.

▽—————▽	A 280 nm
▲—————▲	Alkaline phosphatase
■—————■	Carbohydrate

TABLE V. AMMONIUM SULFATE FRACTIONATION OF M. SODONENSIS
ALKALINE PHOSPHATASE

Fraction	mg ¹ protein ¹	Percent	Units of ² Enzyme	Percent	carbohydrate ¹ μmoles	Percent
G-200 eluate	25.2	100	207	100	64.8	100
40% (NH ₄) ₂ SO ₄ precipitate	0	0	0	0	0.3	0.5%
50% (NH ₄) ₂ SO ₄ precipitate	19.6	77.8	99	47.7	1.92	3.0
70% (NH ₄) ₂ SO ₄ precipitate	3.2	12.3	15	7.4	10.6	16.4
100% (NH ₄) ₂ SO ₄ precipitate	0	0	0	0	.95	1.5
100% Supernatant	2.2	8.4	12	6.0	51.5	79.5

¹Protein and carbohydrate quantitated by the Lowry and the Dubois methods respectively.

²Enzyme activity assayed as described in Materials and Methods with PNPP as substrate.

TABLE VI. PURIFICATION OF M. SODONENSIS ALKALINE PHOSPHATASE

Crude Culture	Enzyme ¹ units	Per cent recovery	mg protein ¹	Specific activity	carbohydrate ¹ μmoles
Supernatant	454.8	100	1,160	0.4	1,972
DEAE-cellulose eluate	228	50.3	49	4.7	60.4
Sephadex G-200 eluate	173	38.1	23	7.5	32.9
50% (NH ₄) ₂ SO ₄ precipitate	93	20.5	7.1	13.1	0.22

¹Analytical methods as described for Table V.

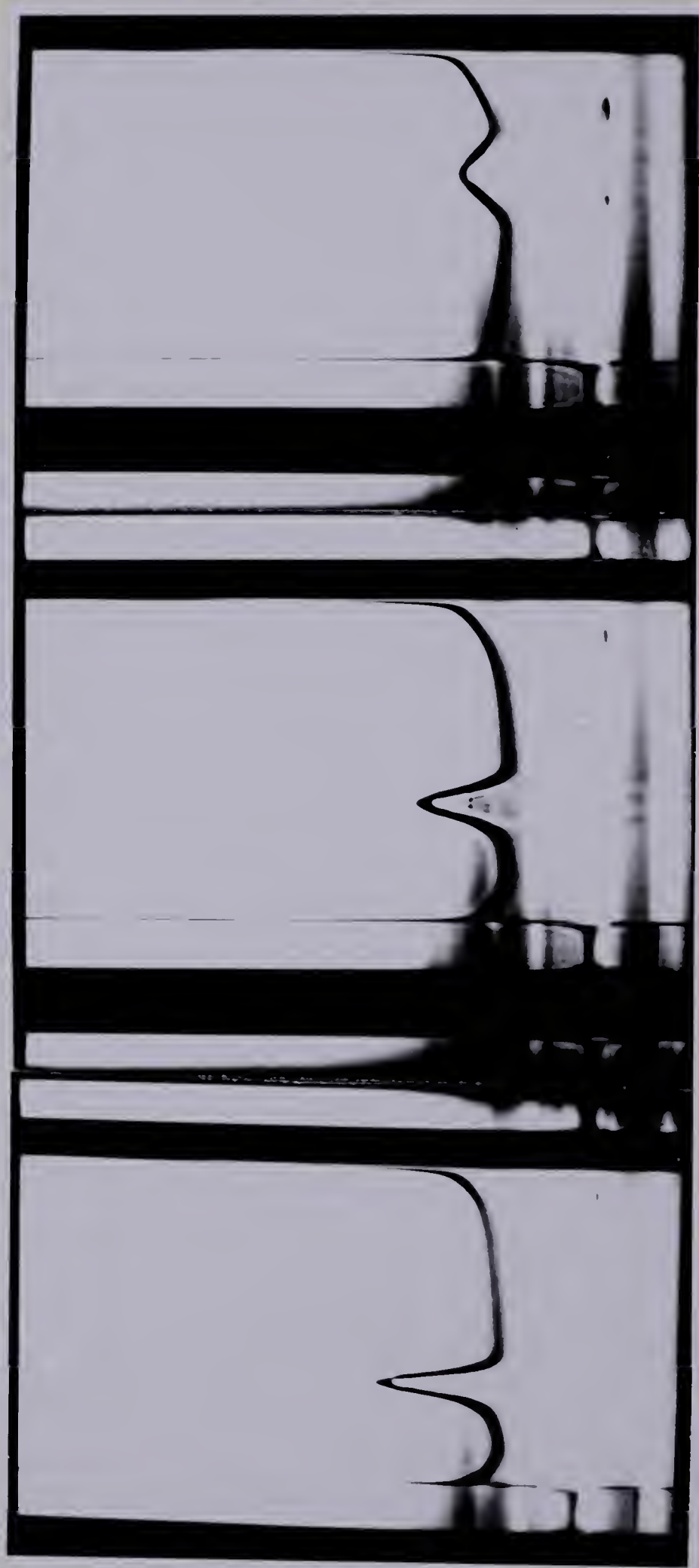


FIGURE 10

SCHLIEREN PATTERN OF M. SODONENSIS ALKALINE PHOSPHATASE

The photographs were taken 6, 38, and 118 minutes after achieving full speed. Sedimentation velocity was measured with a rotor speed of 52,000 rev/min, bar angle 55° , rotor temperature 5° . The $s_{20, w}^{\circ}$ is 4.67.

Gel electrophoresis of the purified enzyme as described in Materials and Methods gave only one broad protein band (Figure 11). The use of phenolphthalein phosphate solution on the gels before staining indicated that this band corresponded to the enzyme activity.

The molecular weight of the alkaline phosphatase was determined using the gel permeation method of Andrews (1964 a, b). A Sephadex G-100 column, 82.5 by 2.5 cm, was equilibrated with 0.1 M KCl - 0.05 M Tris-HCl, pH 7.5. A two ml sample containing Blue Dextran, 0.4 mg lactate dehydrogenase, 3.2 mg bovine serum albumin, 2.5 mg chymotrypsinogen, 2.5 mg ovalbumin, 4 mg γ -globulin, 0.1 mg alkaline phosphatase and 2.5 mg cytochrome c was applied to the column. Two ml fractions were collected. Figure 12 gives the slope obtained when the ratio of the elution volume over the void volume (V_e/V_o) is plotted against the log of the molecular weights of the marker proteins. The molecular weight obtained by this method for M. sodonensis alkaline phosphatase is approximately 55,000 daltons.

2. Substrate Specificity

The substrate specificity of the alkaline phosphatase was tested by substituting the designated substrates in the standard assay mixture at a concentration of 10^{-3} M and following the release of P_i as described in Materials and Methods. The results, summarized in Table VII, show that



FIGURE 11

GEL ELECTROPHORESIS OF PURIFIED M. SODONENSIS
ALKALINE PHOSPHATASE

Two hundred μ grams of purified enzyme were applied to this gel. The gel was allowed to react with phenolphthalein phosphate to visualize the enzyme activity before being fixed and stained with the protein dye, coomassie blue.

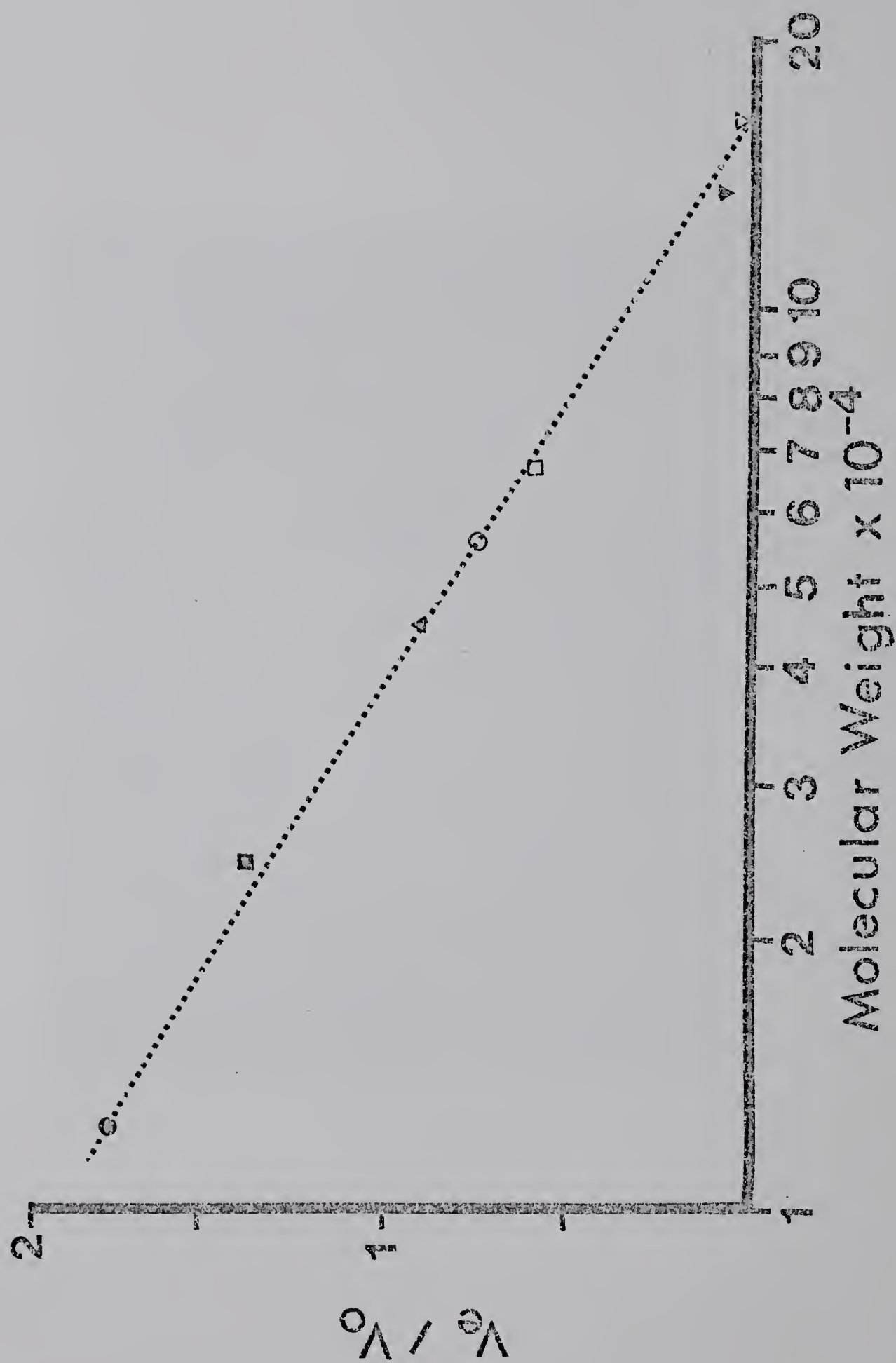


FIGURE 12

MOLECULAR WEIGHT DETERMINATION OF M. SODONENSIS
ALKALINE PHOSPHATASE BY THE GEL PERMEATION METHOD

The molecular weight of the alkaline phosphatase calculated by this method is approximately 55,000 daltons.

- Cytochrome c
- Chymotrypsinogen
- ▲ Ovalbumin
- M.sodonensis alkaline phosphatase
- ▼ Lactate dehydrogenase
- ▽ γ -globulin
- Bovine serum albumin

TABLE VII

SUBSTRATE SPECIFICITY OF M. SODONENSIS ALKALINE PHOSPHATASE

Substrate	Relative Rate of Hydrolysis
Adenosine-5-phosphate	100
Uridine-5-phosphate	87
Thymidine-5-phosphate	85
Cytidine-5-phosphate	94
Guanosine-5-phosphate	96
Deoxyadenosine-5-phosphate	84
Deoxyuridine-5-phosphate	97
Deoxyguanosine-5-phosphate	94
Deoxycytidine-5-phosphate	89
Adenosine-3-phosphate	81
Adenosine-2',3'-cyclic monophosphate	15
Adenosine-3',5'-cyclic monophosphate	2
Adenyl(3'-5') adenosine (ApA)	2
p-nitrophenylthymidine-5'-phosphate	3
bis(p-nitrophenyl) phosphate	8
p-nitrophenyl phosphate	100
Adenosine-5'-monophosphomorpholidate	0
Glucose-6-phosphate	84
Deoxyribose-5-phosphate	100
Riboflavin-5-phosphate	63
Sodium glycerophosphate	82
2-phosphoenolpyruvate	75
Ribose-5-phosphate	75
3-phosphoglyceric acid	84
p-nitrophenylthymidine-3'-phosphate, (T _p N _p)	0

all the nucleotides tested were utilized at similar rates. Glucose-6-phosphate, sodium glycerophosphate, ribose-5-phosphate, 3-phosphoglyceric acid and p-nitrophenyl phosphate were all roughly equivalent as substrates. However, adenosine-2', 3'-cyclic monophosphate, adenosine-3', 5'-cyclic monophosphate, p-nitrophenyl-thymidine-5'-phosphate, bis-(p-nitrophenyl) phosphate, adenylyl (3'-5') adenosine (ApA), and adenosine 5'-monophosphomorpholidate were not utilized indicating a specificity of this enzyme for phosphomonoester and not phosphodiester bonds (Table VII).

3. pH Optimum

The pH activity curve was determined in 0.05 M solutions of buffer in which the pH had been adjusted at 37°. The following buffers were used: KCl-NaOH (pH 12.5, 12.0), glycine-NaOH (pH 10.3, 10.0, 9.8), borate-KCl (pH 10.2, 10.0, 9.8), Tris-HCl (pH 8.8, 8.3, 7.8, 7.2), diethanolamine (pH 9.5, 9.3, 8.6, 8.0), β , β -dimethylglutamate (pH 7.5, 7.0, 6.4), sodium hydrogen malate (pH 6.4, 5.7, 5.3, 4.8). A pH of 10.3 was judged to be optimal (Figure 13) and was employed in assaying the enzyme.

4. Cation Requirements

No cation additions were required for enzyme activity. However, the enzyme was inactivated by 10^{-3} M EDTA indicating that the removal of some cation from the enzyme resulted

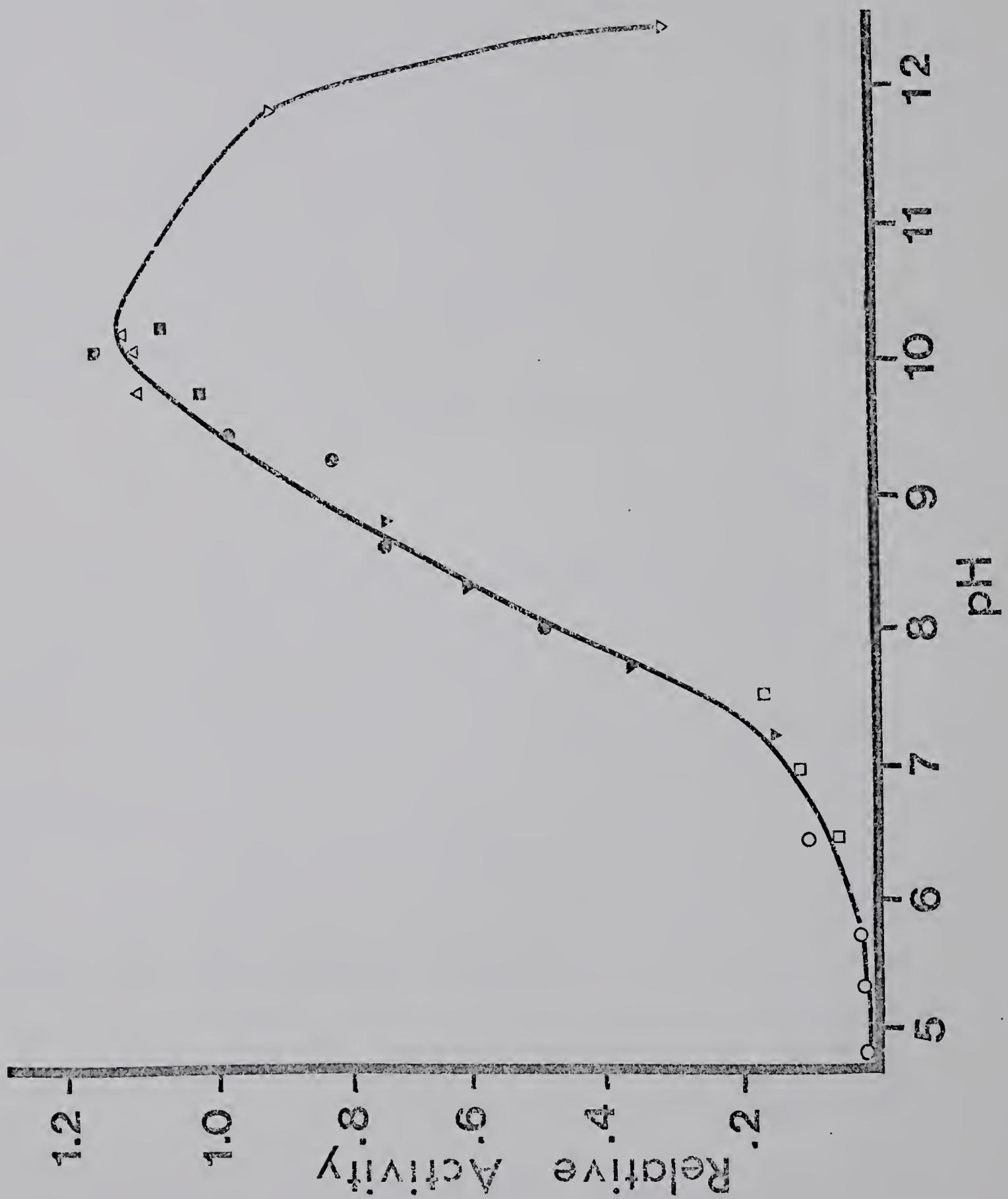


FIGURE 13

pH ACTIVITY CURVE OF M. SODONENSIS ALKALINE
PHOSPHATASE

The following buffers (0.05 M) were used:

- Sodium hydrogen malate
- β , β -dimethylglutamate
- ▼ Tris-HCl
- Diethanolamine
- △ Borate-KCl
- Glycine-NaOH
- ▽ KCl-NaOH

in loss of enzyme activity. The effect of EDTA could be reversed fully with the addition of Ca^{2+} (Table VIII). Co^{2+} could restore only 8% of the activity. The enzyme was inactive in the presence of Ba^{2+} and Fe^{2+} . The enzyme was active to varying degrees in the presence of Mn^{2+} , Zn^{2+} , Mg^{2+} , but none of these cations could reverse to any degree the effect of EDTA. Other chelators were tested and found to be ineffective or much less effective than EDTA in inhibiting the enzyme (Table IX).

Ca^{2+} was also an effective stabilizing agent. Figure 14 shows the rate of inactivation of the enzyme at room temperature in the presence and absence of Ca^{2+} . In the presence of Ca^{2+} , 50% of the activity remained after 160 hours, whereas in its absence this degree of inactivation was reached after 16 hours. The effect of Ca^{2+} is also marked when the enzyme is subjected to higher temperatures. The unprotected enzyme is totally inactive after 6 minutes at 70° whereas the enzyme in the presence of Ca^{2+} retains 83% of its activity (Figures 15, 16). Mg^{2+} did not protect the enzyme from heat inactivation.

TABLE VIII

EFFECT OF DIVALENT CATION ON M. SODONENSIS
ALKALINE PHOSPHATASE ACTIVITY

Cation Addition (μ moles/ml)		EDTA (μ moles/ml)	Per cent activity
Ca^{2+}	0	0	100
	0.42	0	100
	0.85	0	100
	1.7	0	100
	3.4	0	113
	5.1	0	123
Co^{2+}	0	1	0
	1.7	1	100
	0.42	0	88
	0.85	0	89
	1.7	0	84
	3.4	0	89
Mn^{2+}	1.7	1	8
	1.7	0	112
	1.7	1	0
Zn^{2+}	1.7	0	76
	1.7	1	0
Mg^{2+}	1.3	0	49
	1.3	1	0
Fe^{2+}	1.7	0	0
Ba^{2+}	1.7	0	0

TABLE IX

EFFECT OF CHELATING AGENTS ON M. SODONENSIS
ALKALINE PHOSPHATASE ACTIVITY

<u>Chelator</u>	<u>Concentration (M)</u>	<u>% Initial Activity</u>
EDTA	1×10^{-3}	0
Potassium cyanide	1×10^{-3}	137
	2×10^{-3}	106
<u>o</u> -Phenanthroline	1×10^{-3}	96
	2×10^{-3}	86
8-hydroxyquinoline	1×10^{-3}	94
	2×10^{-3}	25
L-cysteine	2×10^{-2}	100
L-histidine	2×10^{-2}	100
L-phenylalanine	8×10^{-3}	100
	4×10^{-2}	128

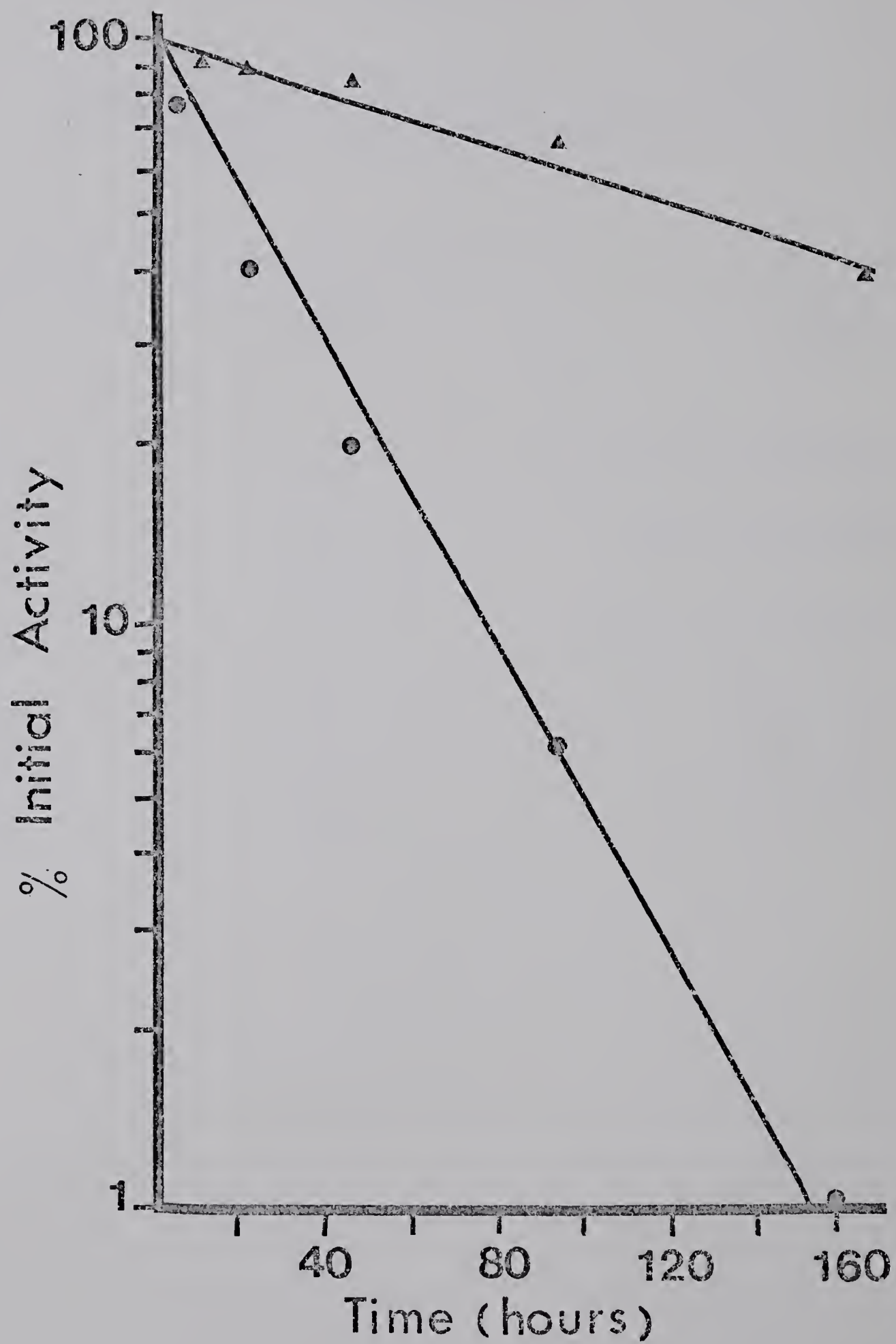
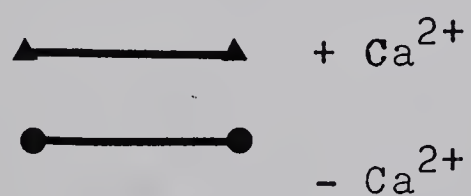


FIGURE 14

EFFECT OF $1.7 \times 10^{-3} \text{ M Ca}^{2+}$ ON THE RATE OF INACTIVATION
OF M. SODONENSIS ALKALINE PHOSPHATASE AT ROOM
TEMPERATURE

Enzyme aliquots were removed at the indicated times. Alkaline phosphatase activity was assayed using PNPP.



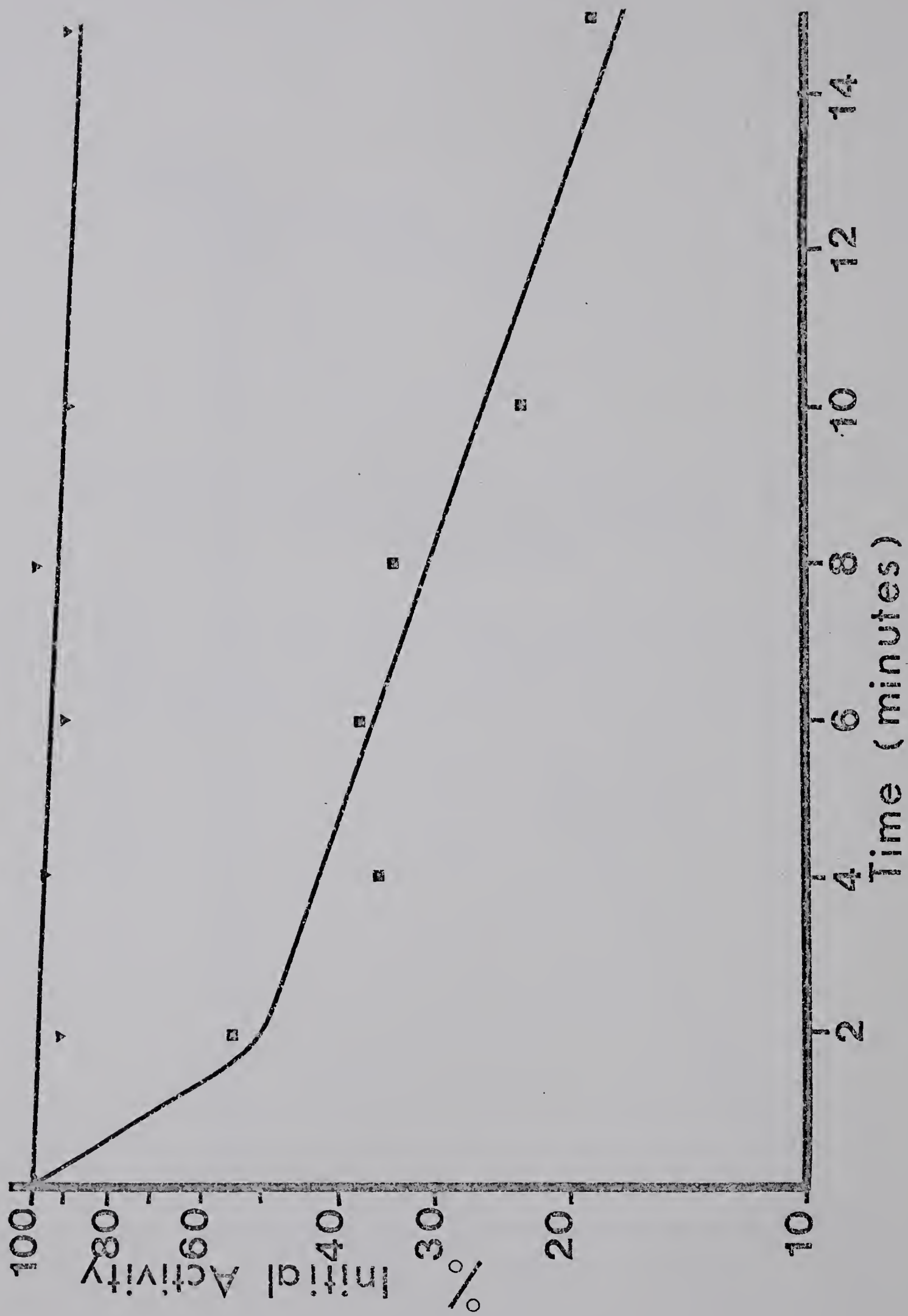
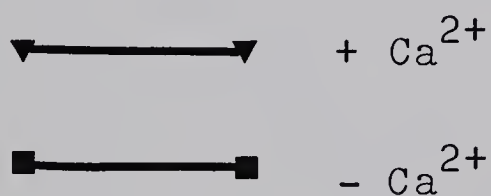


FIGURE 15

EFFECT OF 1.7×10^{-3} M Ca^{2+} ON THE RATE OF INACTIVATION
OF M. SODONENSIS ALKALINE PHOSPHATASE AT 55°

Enzyme aliquots were removed at the indicated times and cooled rapidly in an ethanol-ice bath. Alkaline phosphatase activity was measured using PNPP as substrate.



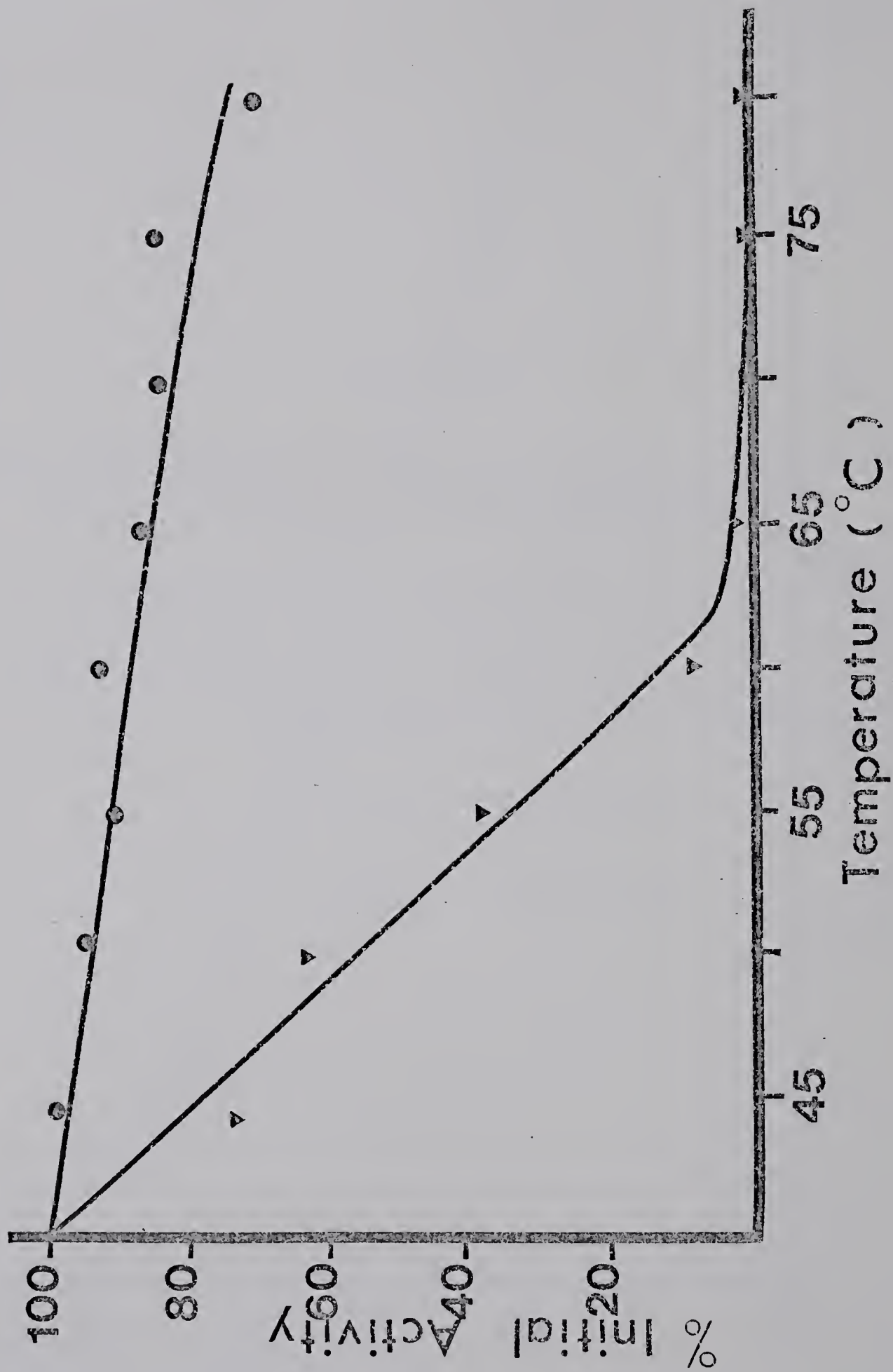
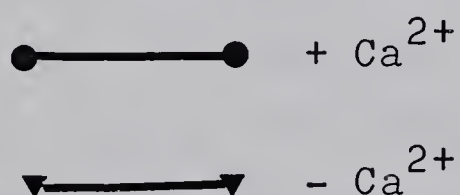


FIGURE 16

EFFECT OF 1.7×10^{-3} M Ca^{2+} ON THE STABILITY OF
M. SODONENSIS ALKALINE PHOSPHATASE AT VARIOUS
TEMPERATURES

The enzyme was incubated for 6 minutes at the temperatures indicated, cooled rapidly in an ethanol-ice bath, and assayed using PNPP as substrate.



5. Kinetic Analyses

A typical kinetic experiment showed that the velocity is proportional to the enzyme concentration between 1.75 and 14.0 μ grams of protein (Figure 17). The K_m for PNPP is 2.44×10^{-5} M and the V_{max} is 65.2 μ moles p-nitrophenol produced per minute per mg protein as determined from a Lineweaver-Burk plot.

The effect of P_i on the hydrolysis of PNPP by the alkaline phosphatase was determined by adding 2.5×10^{-4} to 5.0×10^{-3} M P_i to the standard reaction mixture using 2×10^{-5} to 2×10^{-4} M PNPP as substrate and 0.006 units of purified enzyme. The Lineweaver-Burk plot indicates that P_i is a competitive inhibitor of the reaction (Figure 18). A replot of the slopes from the Lineweaver-Burk plot against P_i concentration gives a K_i value of 3.5×10^{-4} M (Figure 19). If the same experiment was run using ^{14}C -AMP as substrate (5×10^{-7} to 4×10^{-6} M) and phosphate concentrations ranging from 5×10^{-6} to 1.25×10^{-4} M, biphasic inhibition curves are obtained in the Lineweaver-Burk plot (Figure 20), indicating a greater effect of phosphate on the hydrolysis reaction at lower substrate concentrations than at the higher concentration.

The hydrolysis reaction was much more sensitive to arsenate than to P_i . 10^{-3} M arsenate resulted in rapid and complete inhibition of activity when 2×10^{-4} M PNPP was used as substrate.

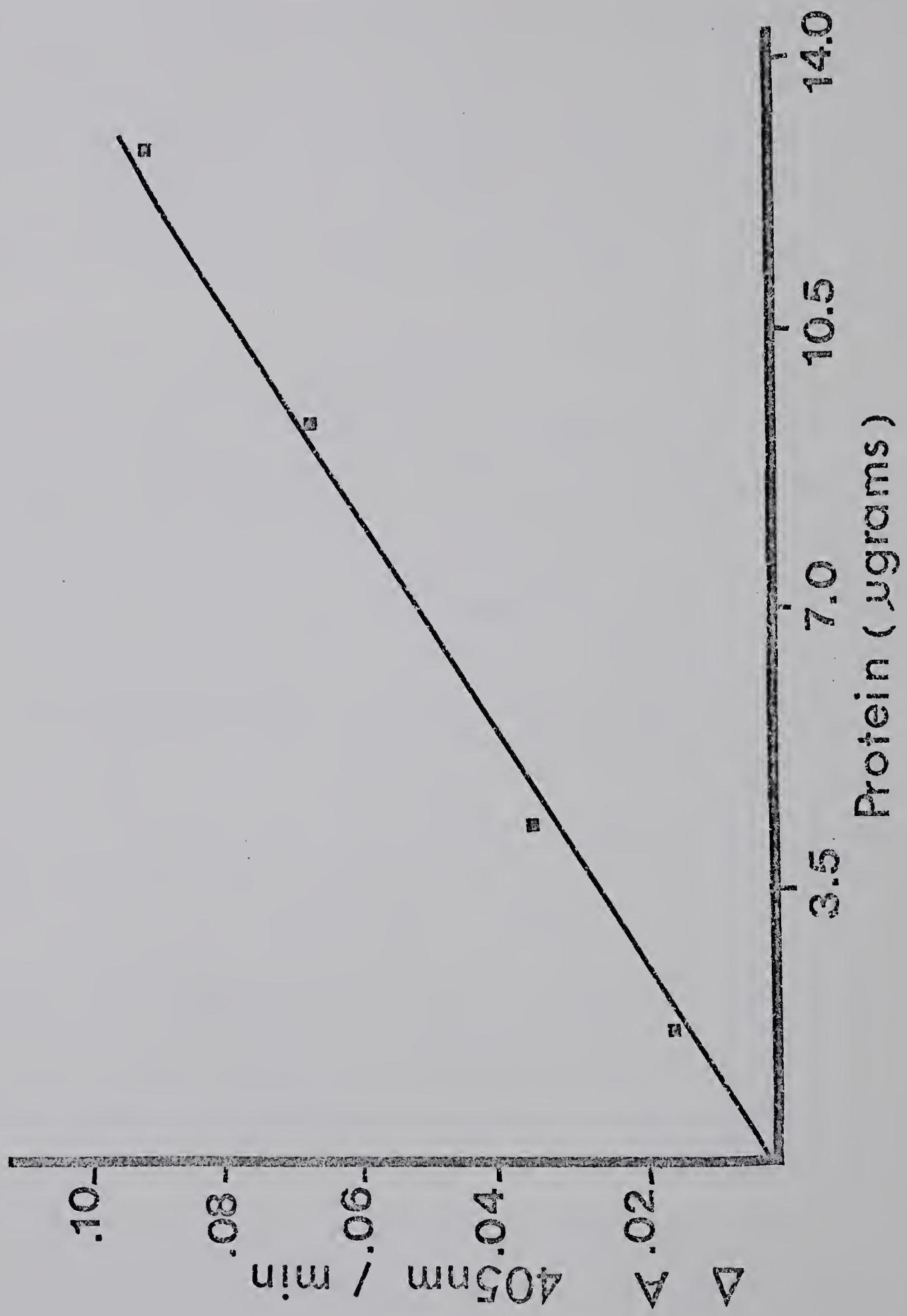


FIGURE 17

EFFECT OF ENZYME CONCENTRATION ON ACTIVITY OF
M. SODONENSIS ALKALINE PHOSPHATASE

0.2 μ moles of PNPP were used as substrate in an assay volume of 1 ml and the change in absorbance was measured at 405 nm.

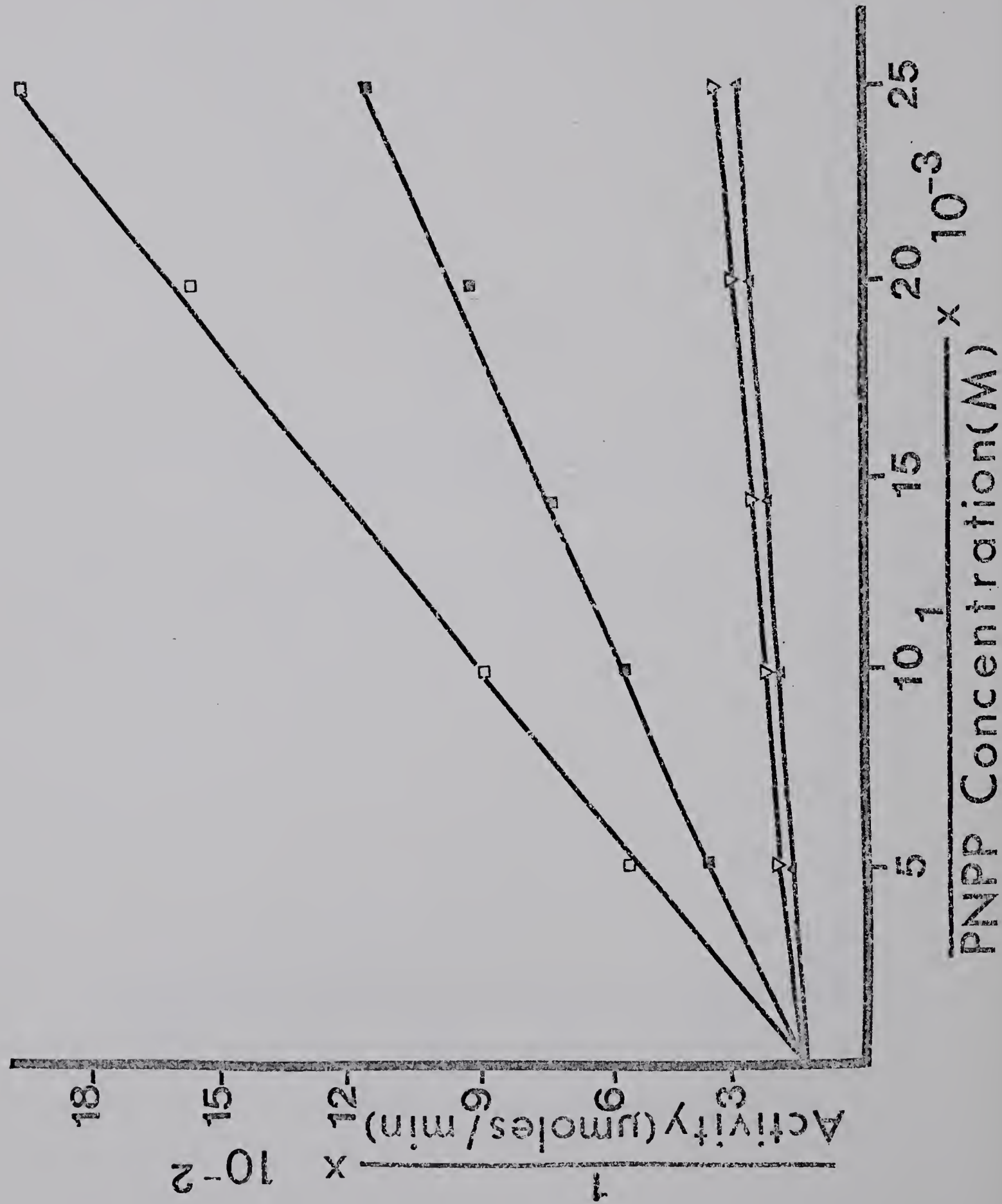

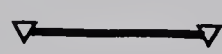




FIGURE 18

INHIBITION BY P_i OF THE HYDROLYSIS OF PNPP BY
M. SODONENSIS ALKALINE PHOSPHATASE

P_i concentrations:

	0
	2.5×10^{-4} M
	2.5×10^{-3} M
	5.0×10^{-3} M

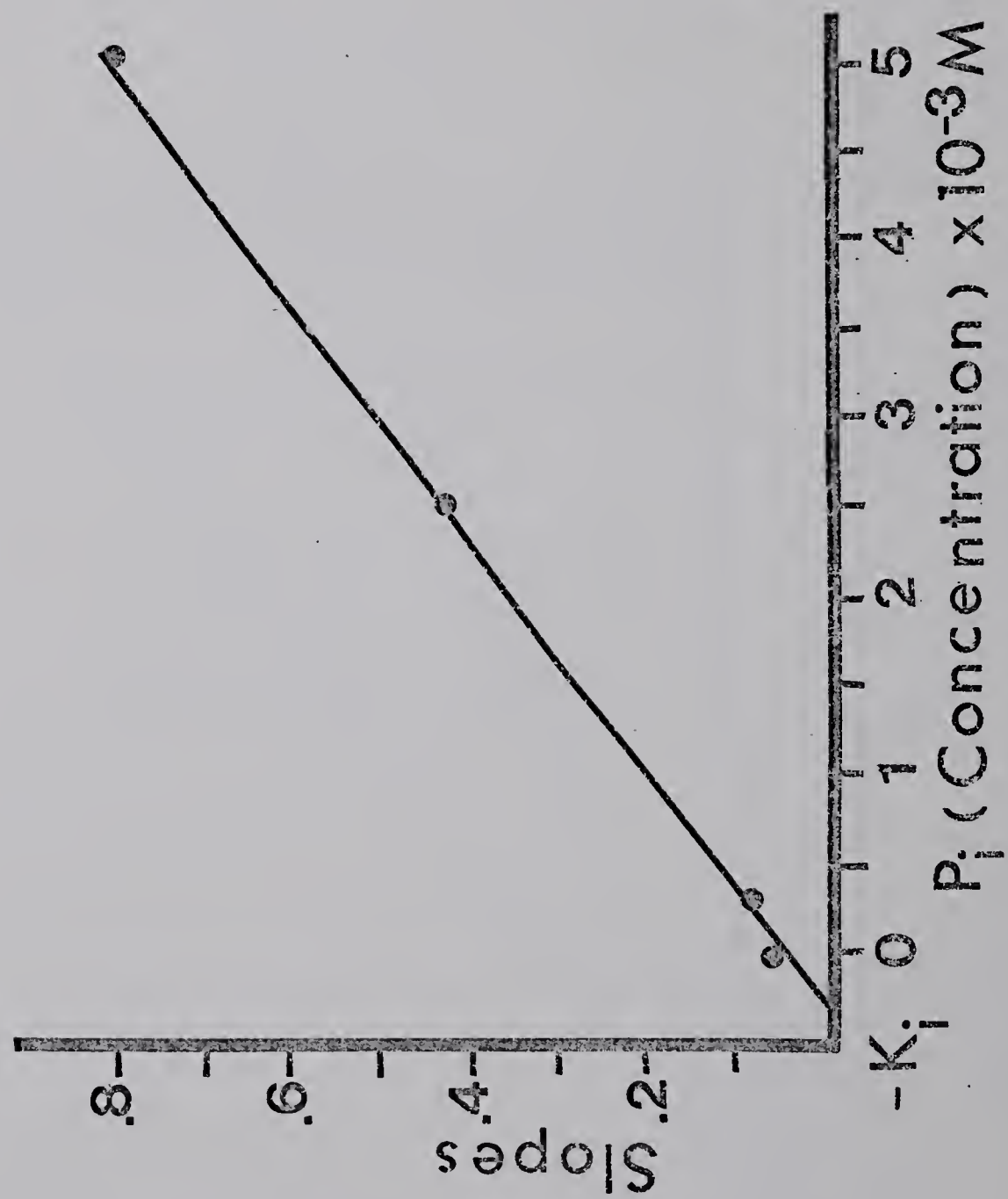


FIGURE 19

REPLOT OF SLOPES OBTAINED IN FIGURE 18 AGAINST P_i
CONCENTRATION

A K_i value of 3.5×10^{-4} M is obtained from the intercept
of the slope with the abscissa.

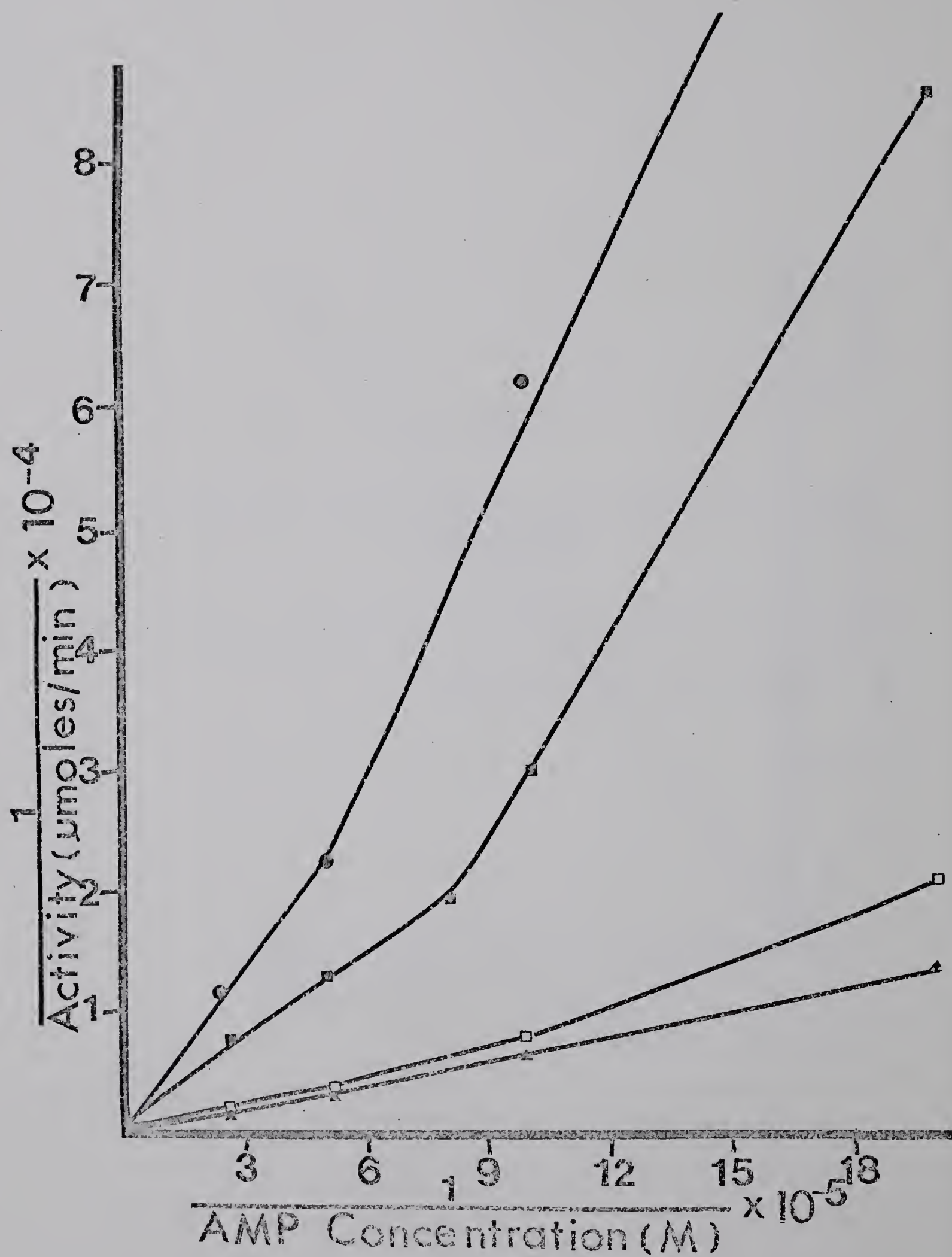






FIGURE 20

INHIBITION BY P_i OF AMP HYDROLYSIS BY M. SODONENSIS
ALKALINE PHOSPHATASE

P_i concentrations:

	0
	$5 \times 10^{-6} \text{ M}$
	$7.5 \times 10^{-5} \text{ M}$
	$1.25 \times 10^{-4} \text{ M}$

6. Transphosphorylase Activity

The presence of transphosphorylase activity in the purified alkaline phosphatase was assayed as indicated in Materials and Methods. As shown in Figure 21, addition of alkaline phosphatase to the assay mixture containing PP_i resulted in an marked increase in NADPH production over the control to which no alkaline phosphatase was added. Similar results were obtained with AMP in the reaction mixture instead of PP_i . The transphosphorylation reaction depended on the addition of Ca^{2+} to the assay mixture. The addition of a similar amount of Ca^{2+} (1.7 mM) did not change the rate of PNPP hydrolysis by the enzyme, indicating a higher Ca^{2+} requirement for the transphosphorylase reaction than for the phosphomonoesterase reaction.

As expected the transphosphorylase activity of the enzyme resulted in marked activation of the enzyme by increasing concentrations of Tris. As indicated in Figure 22, up to an eight-fold increase in activity resulted from the addition of Tris. Similar concentrations of sucrose or NaCl did not markedly affect enzyme activity.

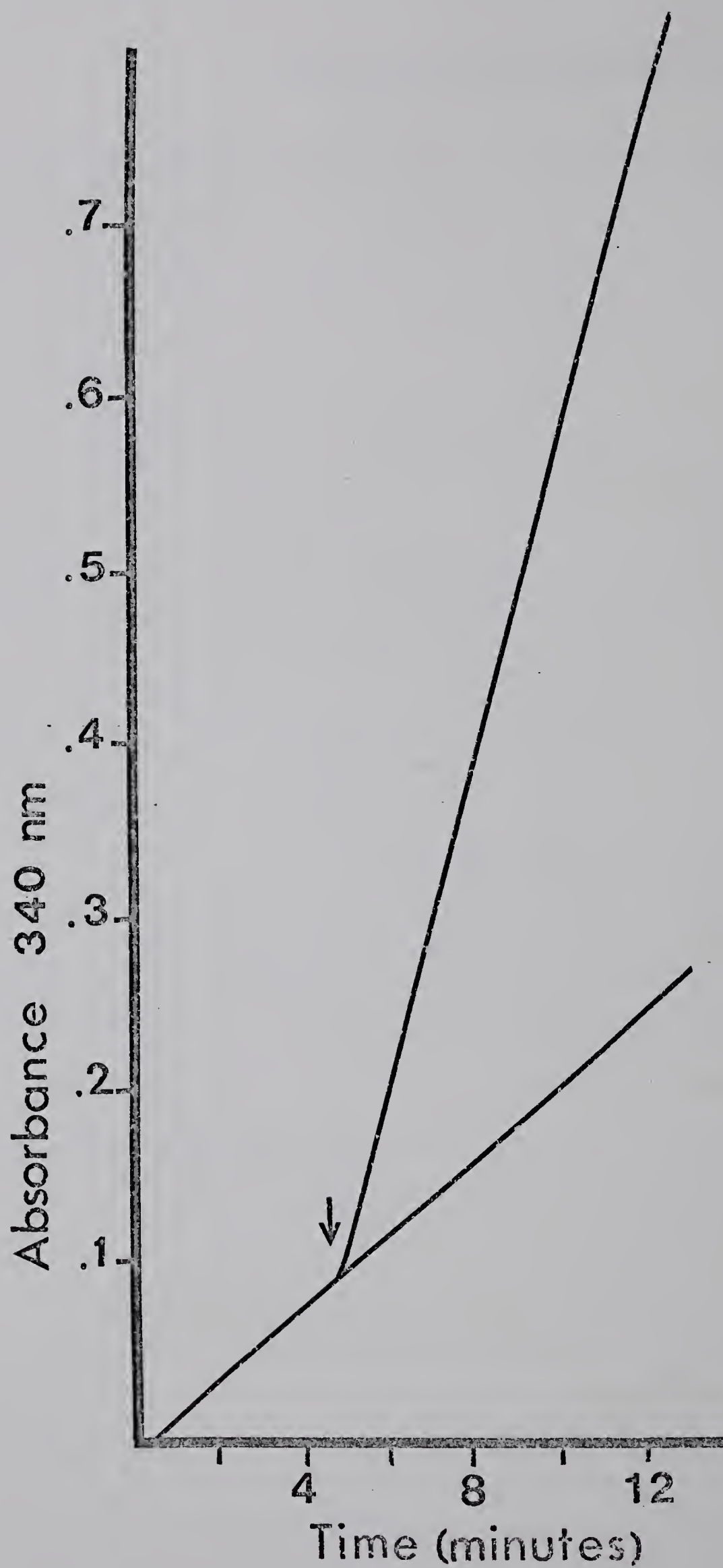


FIGURE 21

TRANSPHOSPHORYLASE ACTIVITY OF M. SODONENSIS
ALKALINE PHOSPHATASE

The assay was done as indicated in Materials and Methods, using P_i in the reaction mixture. At five minutes, indicated by the arrow, alkaline phosphatase was added to one cuvette and production of NADPH, as measured by the increase in absorbance at 340 nm, was followed using a recording spectrophotometer.

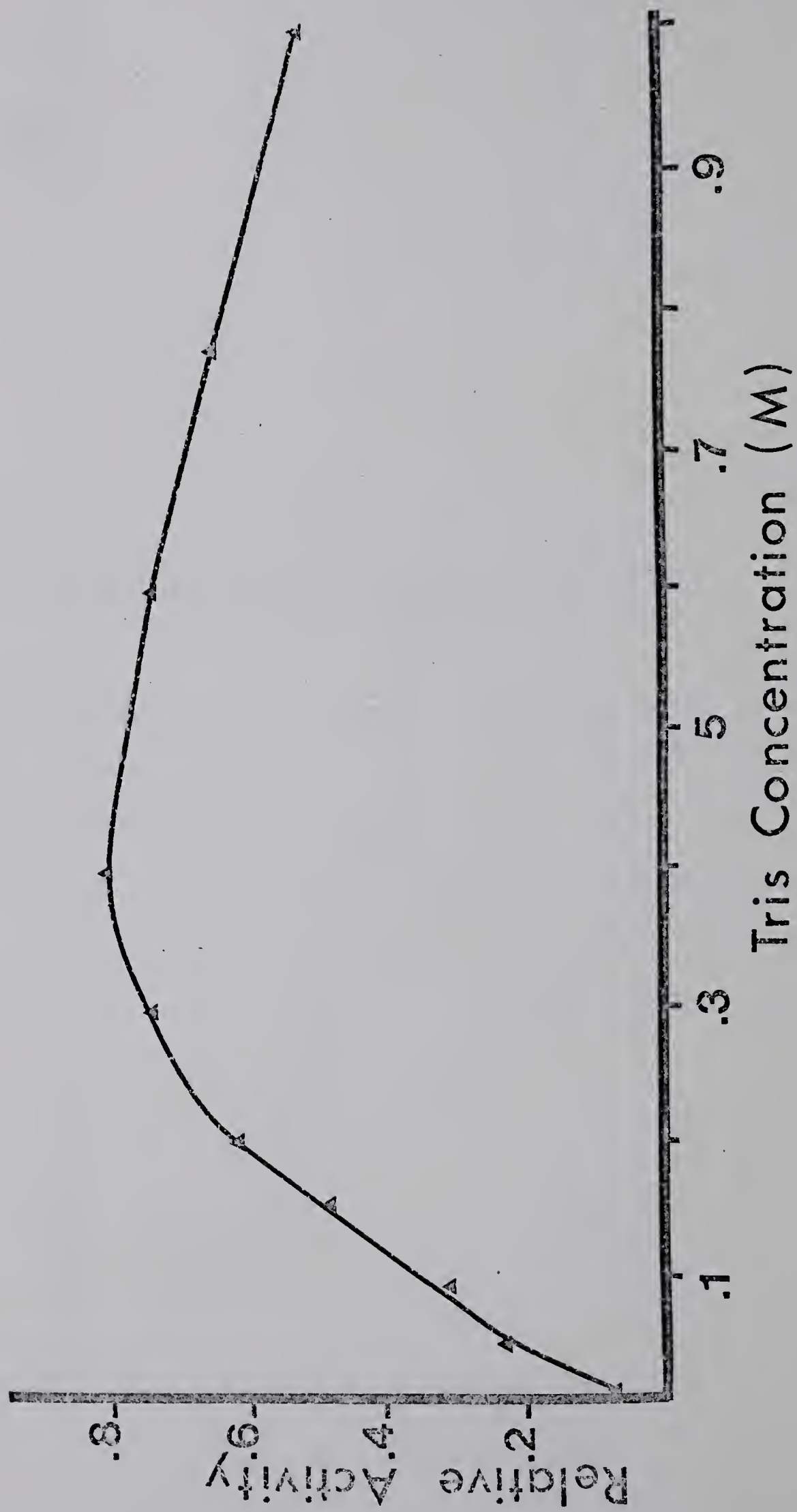


FIGURE 22

EFFECT OF INCREASING CONCENTRATIONS OF TRIS ON
M. SODONENSIS ALKALINE PHOSPHATASE ACTIVITY

The assays were done in Tris-HCl buffer, pH 8.9, instead of the usual Glycine-NaOH buffer, pH 10.3.

7. Enzyme Inhibition.

M. sodonensis alkaline phosphatase was incubated for thirty minutes at 37° with a number of protein inactivating agents. Then the enzyme reaction was initiated by the addition of $0.2\ \mu\text{moles}$ ^{14}C -AMP to give a total assay volume of $0.1\ \text{ml}$. The release of ^{14}C -adenosine was determined as indicated in Materials and Methods. The results obtained are presented in Table X. Although some of the reagents affecting SH-groups are ineffective against the enzyme, the effectiveness of some others indicates that an SH-group is important for enzyme activity. The other protein reagents tested proved to have no effect on enzyme activity except for dansyl chloride. It is a reagent of broad specificity and therefore its effect on alkaline phosphatase does not reveal which amino group(s) is (are) important for catalytic activity.

TABLE X. EFFECT OF SOME ENZYME INHIBITORS ON M. SODONENSIS ALKALINE
PHOSPHATASE ACTIVITY¹

Inhibitor	Concentration(M)	% initial activity	Amino acid group
Mersalyl acid	1 x 10 ⁻² 5 x 10 ⁻³	48 45	-SH
Methylmaleimide	1 x 10 ⁻² 5 x 10 ⁻³	101 98	-SH
p-chloromercuribenzoate	3.5 x 10 ⁻³ 1 x 10 ⁻³	55 53	-SH
Iodoacetate	1 x 10 ⁻² 5 x 10 ⁻³	88 108	-SH, His
Mercuric chloride	1 x 10 ⁻⁴	74	-SH
N-Bromosuccinimide	1 x 10 ⁻² 5 x 10 ⁻³	100 99	His, Trp, Tyr, Cys
5,5'-dithiobis-(-2-nitro- benzoic acid)	7 x 10 ⁻⁴ 2 x 10 ⁻⁴	101 91	-SH
5-aminotetrazole	1 x 10 ⁻² 5 x 10 ⁻³	123 96	
Sodium azide	1 x 10 ⁻² 5 x 10 ⁻³	103 97	amino, Lys, Tyr, Cys
Dansyl chloride	2.5 x 10 ⁻³	51	free amino, Tyr, Ser Cys

¹Details of assay procedures are given in the text.

III. KINETIC STUDY OF THE 5'-NUCLEOTIDASE

1. Purification

The 5'-nucleotidase was purified as described in Materials and Methods using 16 liters of culture supernatant from M. sodonensis cells grown in TCS broth. One hundred μgm of the purified enzyme was used for the disc gel electrophoresis as described in Materials and Methods. The purified enzyme was not free of diesterase activity and gave two bands on acrylamide gel electrophoresis (see gel 4, Figure 2). The proteins ran toward the anode (+) and the leading protein band was only 1 mm behind the riboflavin band. Several gels were cut into sections and the enzyme in the sections eluted with 0.01 M Tris-HCl buffer, pH 8.8 for 18 hours. Comparison of stained gels with the activity obtained in the sections indicated that the leading band possessed both 5'-nucleotidase and diesterase activity and that the slower band had only diesterase activity.

2. Binding of Products to the Enzyme

The reaction mixture consisted of 5'-nucleotidase (15 μgm), 0.01 M Tris, pH 8.8, and, in the case of the test sample, 8×10^{-3} M P_i all in a final volume of 0.2 ml. The

reaction mixtures were incubated at 65° and 0.02 ml aliquots were removed at the stated times and cooled quickly in an ethanol-ice bath. Equivalent amounts of P_i were added to the control samples immediately prior to assaying for 5'-nucleotidase activity using the ^{14}C -AMP assay procedure. The experiment was repeated using 6.25×10^{-4} , 2.5×10^{-3} , and 1.25×10^{-2} M adenosine and an incubation temperature of 71° . The results shown in Figures 23 and 24 indicate that both adenosine and P_i protected the enzyme from heat inactivation but that adenosine was more effective in protecting the enzyme against heat inactivation than was P_i .

Both P_i and adenosine also protected the enzyme from inactivation by a number of reagents known to combine with proteins. The enzyme was incubated with 0.01 M Tris-HCl buffer, pH 8.8, 10^{-3} M histidine, and 1.7×10^{-3} M Mn^{2+} . Adenosine (2.5×10^{-3} M) or P_i (8×10^{-3} M) were added to the appropriate samples which were incubated for 30 minutes at 37° . Then the reagents were added to yield the final concentrations specified (Table XI), the incubation prolonged for an additional 30 minutes, and the activity assayed with the ^{14}C -AMP technique. The control samples were those containing the protein reagents but no P_i or adenosine, and the activity found in these control samples and in the test samples was expressed as per cent of the activity found in samples without the protein reagents but with equivalent amounts of P_i or adenosine. The results are given in Table XI. Adenosine protected the enzyme from

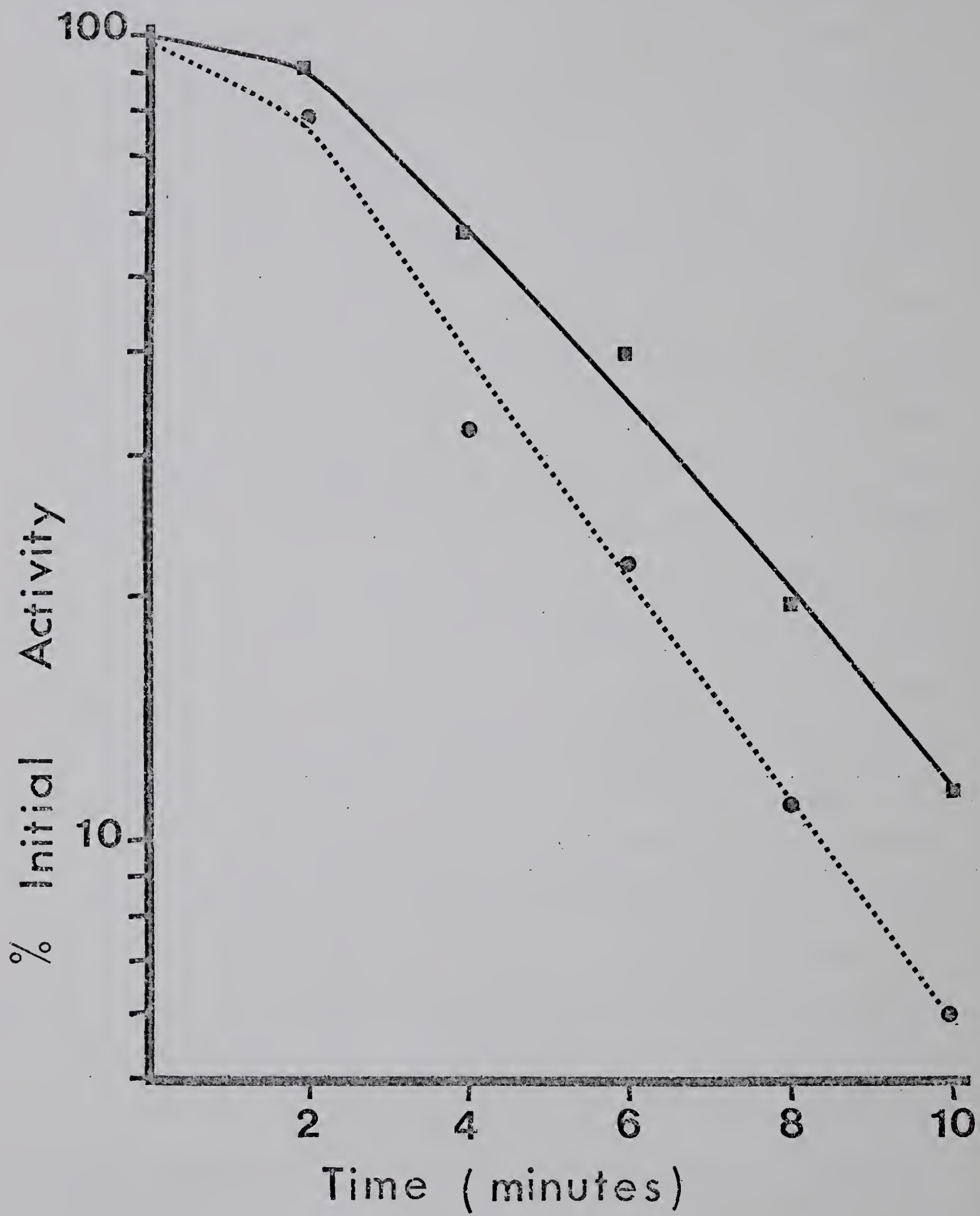
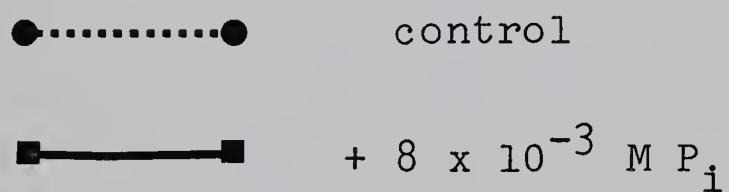


FIGURE 23

EFFECT OF P_i ON HEAT INACTIVATION OF M. SODONENSIS
5'-NUCLEOTIDASE AT 65°

5'-Nucleotidase activity was assayed using the ^{14}C -AMP technique.



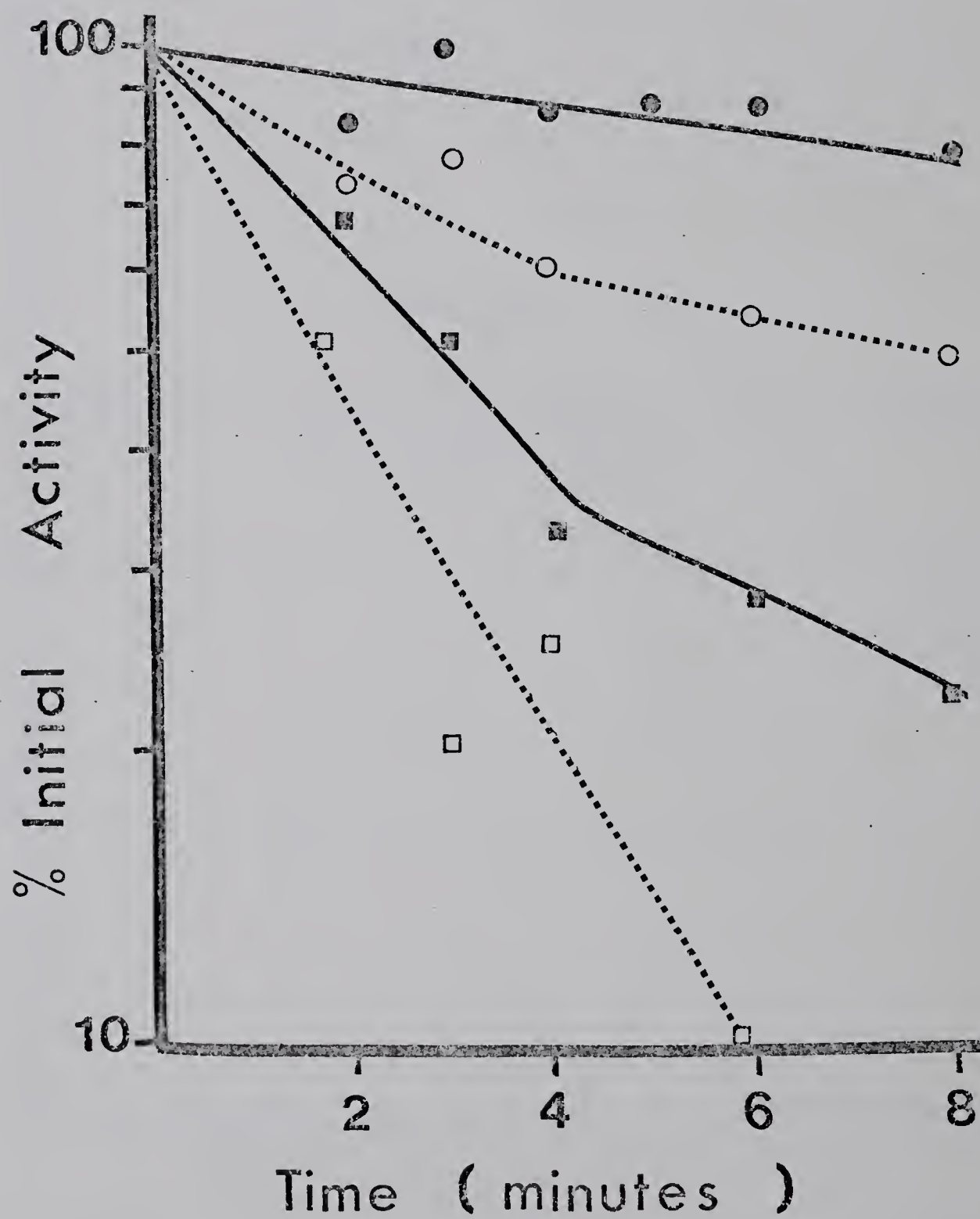


FIGURE 24

EFFECT OF ADENOSINE ON HEAT INACTIVATION OF
M. SODONENSIS 5'-NUCLEOTIDASE AT 71°

5'-Nucleotidase activity was assayed using the ^{14}C -AMP technique.

□.....□	Control
■————■	+ 6.25×10^{-4} M adenosine
○.....○	+ 2.5×10^{-3} M adenosine
●————●	+ 1.25×10^{-2} M adenosine

TABLE XI. EFFECT OF SOME ENZYME INHIBITORS ON M. SODONENSIS
5'-NUCLEOTIDASE ACTIVITY

Protein Reagent	Concentration(M)	% Initial Activity		
		Adenosine ($2.5 \times 10^{-3}M$)	P_i ($8 \times 10^{-3}M$)	Control
2-hydroxy-5-nitrobenzyl bromide	1.5×10^{-3}	73	-	80
	3×10^{-3}	77	-	74
	7.5×10^{-3}	58	80	82
2-chloro-3,5-dinitro- pyridine	1×10^{-3}	92	-	82
	2×10^{-3}	78	80	82
	1×10^{-2}	100	-	68
<u>N</u> -acetylimidazole	2×10^{-2}	95	65	56
Trinitrobenzoic sulfonic acid	1.5×10^{-3}	66	-	63
	3×10^{-3}	66	47	68
	5×10^{-4}	96	-	86
	1×10^{-3}	80	-	75
Dansyl chloride	2.5×10^{-3}	66		77
	5×10^{-3}	26	51	21
	1×10^{-2}	9	20	5

TABLE XI, continued

Protein Reagent	Concentration(M)	% Initial Activity		
		Adenosine (2.5x10 ⁻³ M)	P _i (8x10 ⁻³ M)	Control
<u>p</u> -hydroxymercuribenzoate	5x10 ⁻³	99	96	91
	1x10 ⁻²	75	102	96
<u>N</u> -bromosuccinimide	5x10 ⁻³	89	89	85
	1x10 ⁻²	64	69	65
<u>p</u> -chloromercuribenzoate	1x10 ⁻³	105	102	85
Dithiothreitol	5x10 ⁻⁴	79	105	114
	1x10 ⁻³	121	107	119

inactivation by N-acetylimidazole and P_i from inactivation by 1,5-difluoro-2,4-dinitrobenzene. P_i like adenosine also protected the enzyme against the effect of p-chloromercuribenzoate.

Equilibrium dialysis of the enzyme was carried out against 0.01 Tris-HCl, pH 8.8, containing 3×10^{-7} M ^{14}C -adenosine and different amounts of non-radioactive adenosine to a maximum total final concentration of 4.2×10^{-6} M adenosine. Equilibrium dialysis studies were carried out in 1 ml capacity equilibrium dialysis cells (Chemical Rubber Co., Inc.) or using 1 ml of enzyme (0.56 mg) in a dialysis bag in 50 ml of the above Tris-adenosine solution. The results were the same with both techniques. In both cases dialysis was carried out with continuous stirring with magnetic stirrers, at room temperature for 18 hours, at which time equilibrium had been achieved. The enzyme activity was stable under these conditions. A Scatchard plot (Figure 25) gave an r value of approximately 7 μmoles of adenosine bound per gm of enzyme or 3.5 moles of adenosine per mole of enzyme (assuming a molecular weight of 500,000 daltons (Berry, et al, 1970)). Binding did not occur in 0.05 M Tris nor did it occur at 5° . Binding occurred normally when 0.01 M Tris was replaced with deionized water. The presence of P_i (10^{-5} - 10^{-4} M) had no effect on the binding of ^{14}C -adenosine by the enzyme.

The binding of $^{32}\text{P}_i$ to the enzyme was shown by putting a sample (0.7 ml) containing $^{32}\text{P}_i$ (0.2 nM, 2 μcurie) and

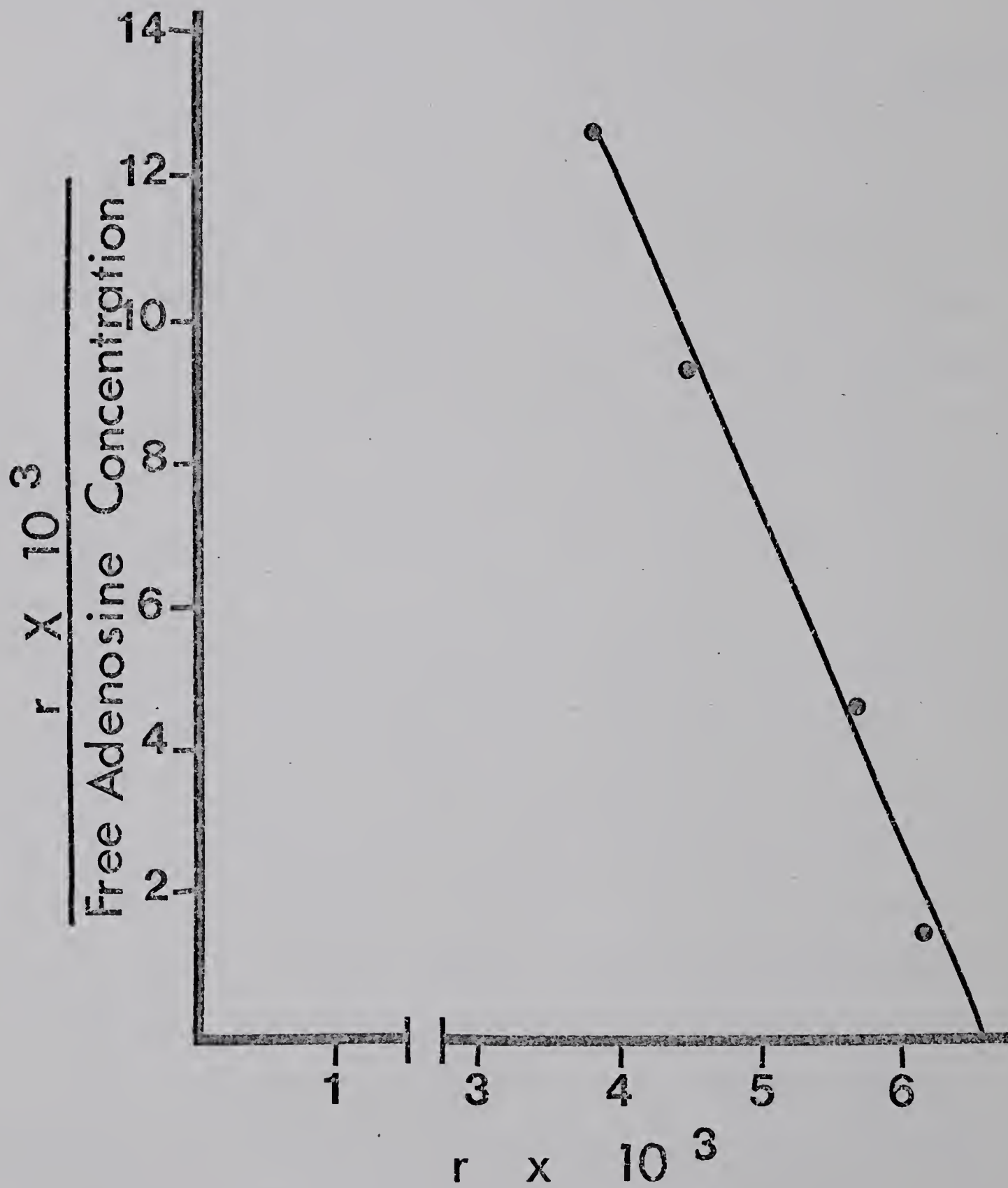


FIGURE 25

SCATCHARD PLOT SHOWING BINDING OF ^{14}C -ADENOSINE TO
M. SODONENSIS 5'-NUCLEOTIDASE

'r' equals μmoles of adenosine bound to the enzyme per mg of protein. The 'free adenosine concentration' is expressed as μmoles of adenosine.

enzyme (380 μ gm) on a G50 Sephadex column (27 cm x 1 cm) equilibrated with 0.01 Tris, pH 8.8, and testing the eluate for enzyme activity and radioactivity (Figure 26). Some $^{32}\text{P}_i$ was eluted with the enzyme further confirming the ability of the enzyme to bind P_i .

3. Product Inhibition by P_i

Product inhibition by P_i was carried out at AMP concentrations ranging from 2.5×10^{-7} to 4×10^{-4} M. The P_i concentrations ranged from 10^{-5} - 10^{-2} M. The assays were carried out using the ^{14}C -AMP technique and adding P_i with the enzyme and the assay mixture 30 minutes before initiating the reaction by the addition of the substrate, ^{14}C -AMP.

At substrate concentrations ranging from 5×10^{-7} to 4×10^{-6} M, the phosphate inhibition appears to be competitive (Figure 27). With higher substrate concentrations (5×10^{-6} to 4×10^{-5} M), the lower P_i concentrations gave both slope and intercept effects whereas the higher concentrations of P_i had simply intercept effect (Figure 28). This suggests that the effect obtained at the higher substrate concentrations may simply be due to dead-end binding of P_i to some enzyme form at those concentrations. The nonspecificity of the inhibition is also suggested by the fact that older preparations of the 5'-nucleotidase become progressively less sensitive to P_i inhibition, requiring

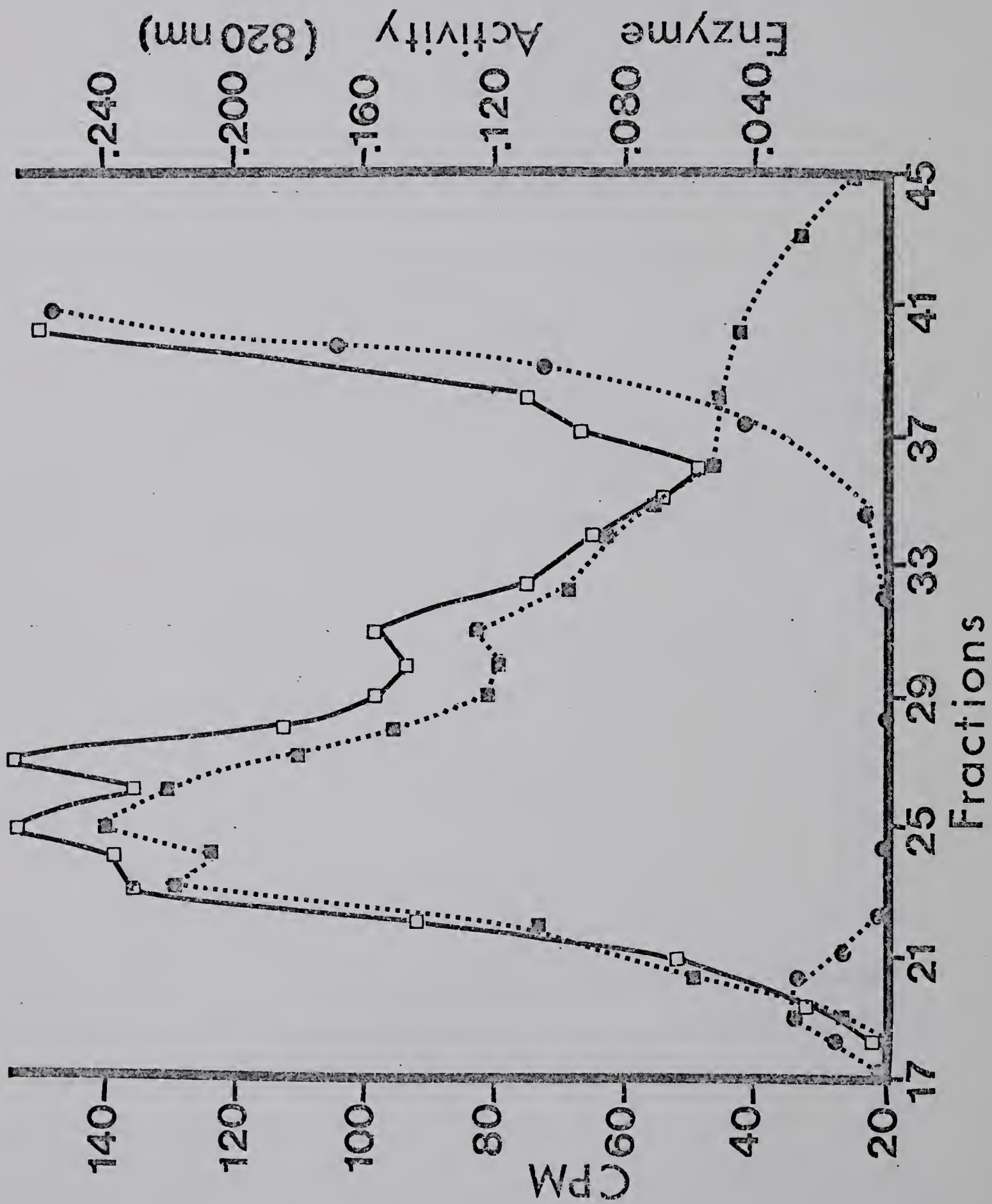
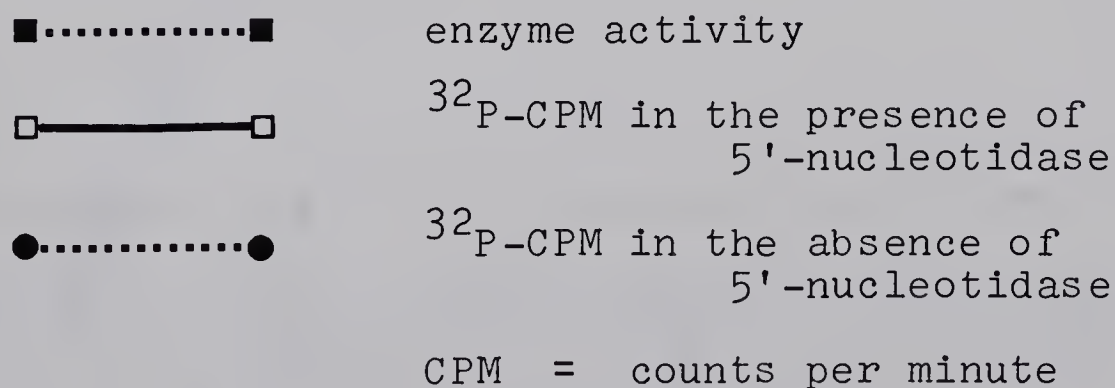


FIGURE 26

ELUTION PROFILE OF M. SODONENSIS 5'-NUCLEOTIDASE
AND $^{32}\text{P}_i$ FROM SEPHADEX G-50

$^{32}\text{P}_i$ and 5'-nucleotidase (as indicated in the text) were applied to a Sephadex G-50 column. Enzyme activity was assayed by measuring the release of P_i from AMP using 5 μ liter of each fraction. A similar volume of each fraction was assayed for P_i and the value obtained was subtracted from that obtained from the enzymatic assay. 100 μ liters were used for the determination of the $^{32}\text{P}_i$. In a separate experiment, a similar amount of $^{32}\text{P}_i$ was eluted from the column to determine the amount of polyphosphates present in the radioactive preparation. The volume of each fraction was 300 μ liters.



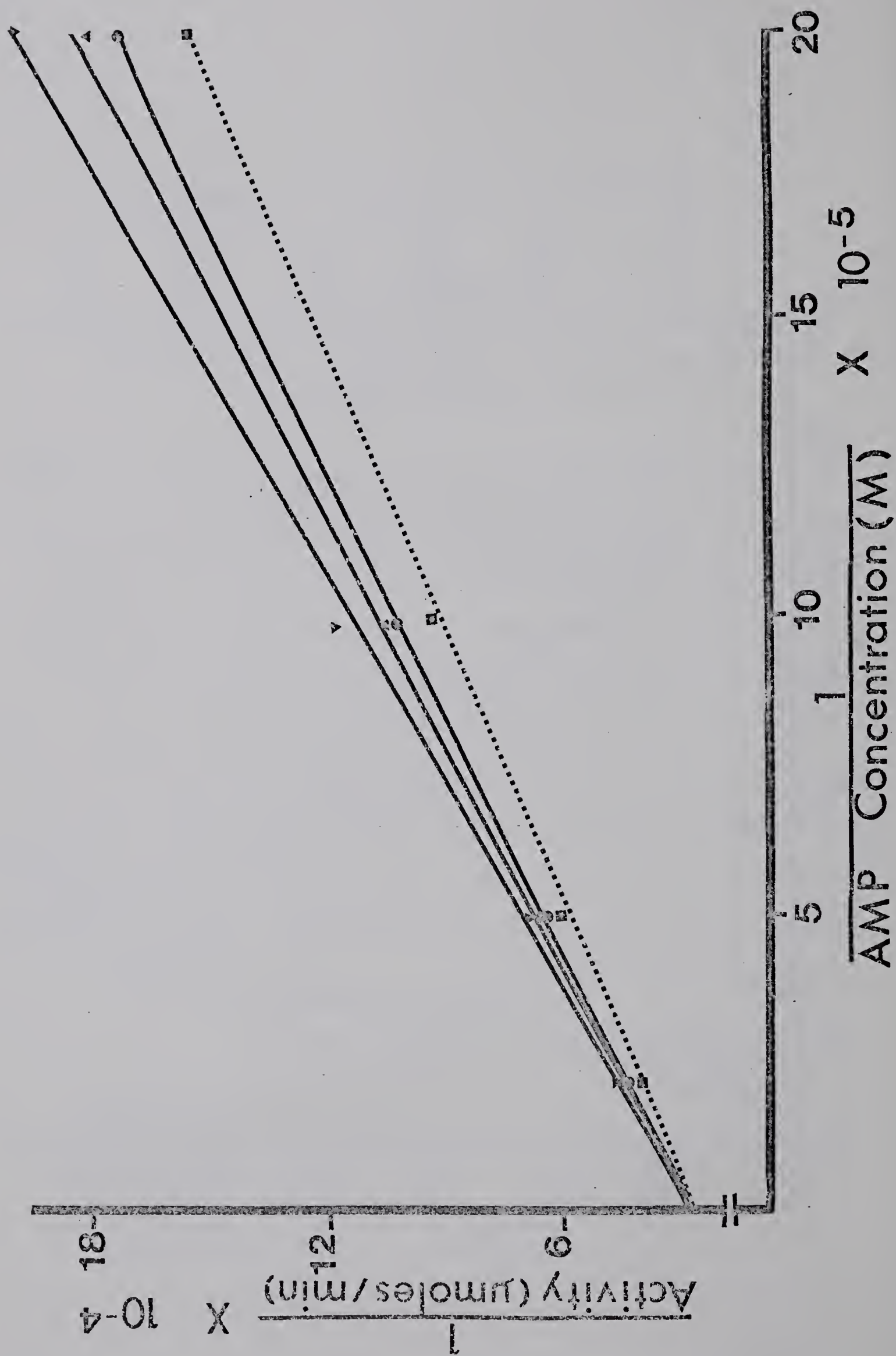
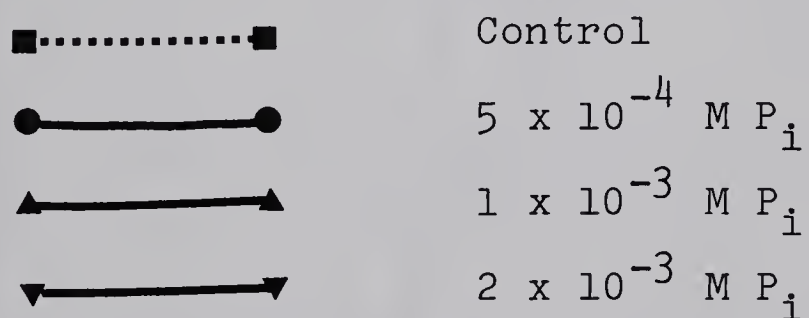


FIGURE 27

LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF P_i ON
M. SODONENSIS 5'-NUCLEOTIDASE ACTIVITY AT LOW
 CONCENTRATIONS

The AMP concentrations used ranged from 5×10^{-7} to 4×10^{-6} M. The ^{14}C -AMP assay was used.



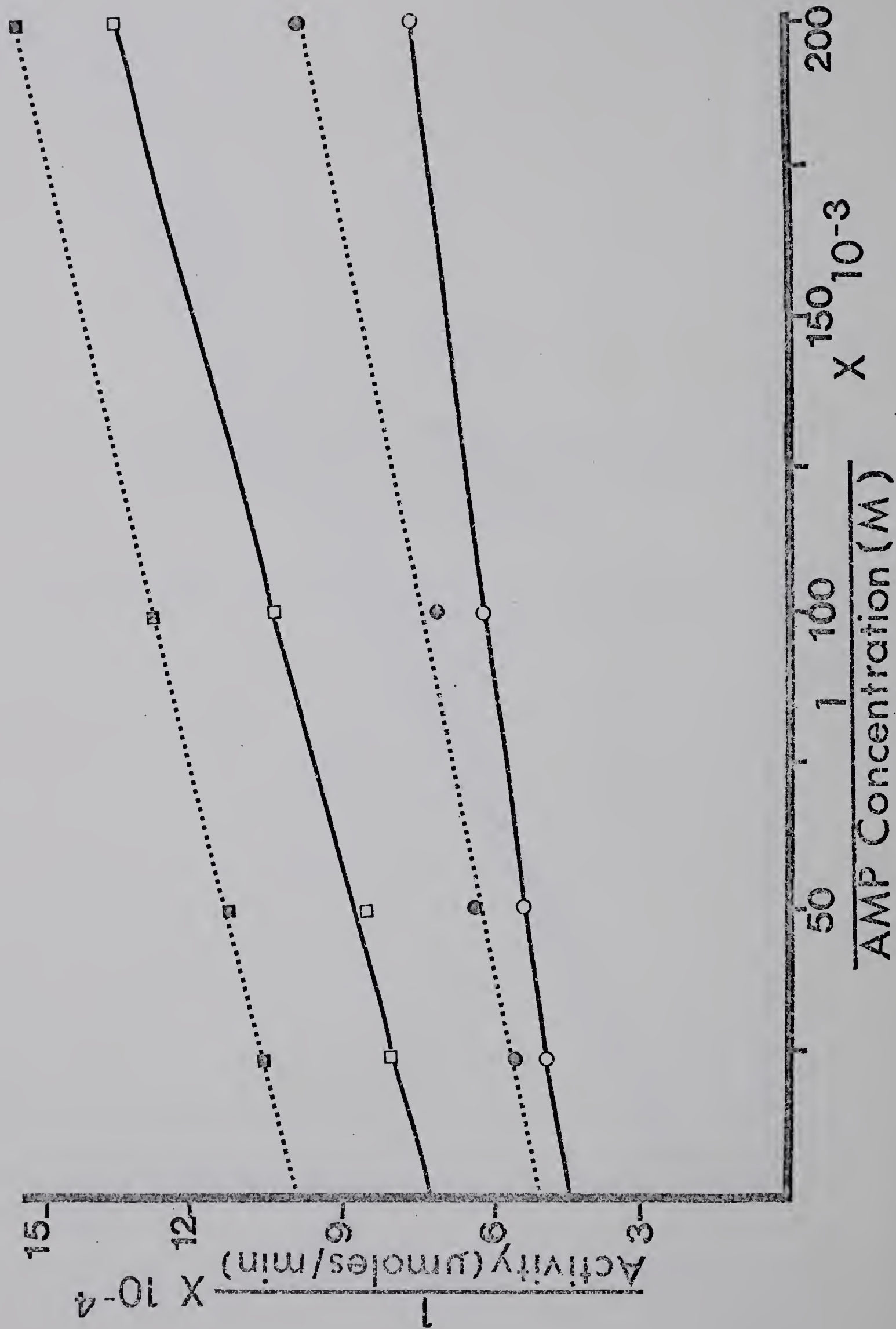


FIGURE 28

LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF P_i ON
M. SODONENSIS 5'-NUCLEOTIDASE ACTIVITY AT HIGHER
SUBSTRATE CONCENTRATIONS

The AMP concentrations used ranged from 5×10^{-6} to
 4×10^{-5} M. The ^{14}C -AMP assay was used.

○————○	Control
●.....●	8×10^{-4} M P_i
□————□	3.2×10^{-3} M P_i
■.....■	8×10^{-3} M P_i

higher concentrations of P_i to achieve an equivalent amount of inhibition, and demonstrating only competitive inhibition.

4. Product Inhibition by Nucleosides

In like manner, inhibition of AMP hydrolysis by adenosine was tested over a large range of nucleoside concentrations (1×10^{-5} to 5×10^{-3} M) and the results plotted in a Lineweaver-Burk plot (Figure 29). The lower concentrations of adenosine caused only slight changes in slope but more considerable changes in intercept. The lines are almost parallel indicating apparent uncompetitive inhibition. At higher concentrations of adenosine (i.e. above 1×10^{-4} M), the inhibition becomes noncompetitive (Figure 29). The same inhibition pattern was obtained when 100-fold lower substrate concentrations were used. Replots of intercepts and slopes versus adenosine concentration give hyperbolic slopes (Figure 30). When $\frac{1}{\text{slope}_i - \text{slope}_0}$ or $\frac{1}{\text{intercept}_i - \text{intercept}_0}$ is plotted against $1/I$ (where slope_i is the slope at the inhibitor concentration I , and slope_0 is the slope when $I = 0$, and the same for the intercepts), a hyperbolic inhibition will give a straight line, since the hyperbola is identical in form to the one describing the initial rate of an enzyme-catalyzed reaction if the horizontal axis is raised so that the curve goes through the origin.

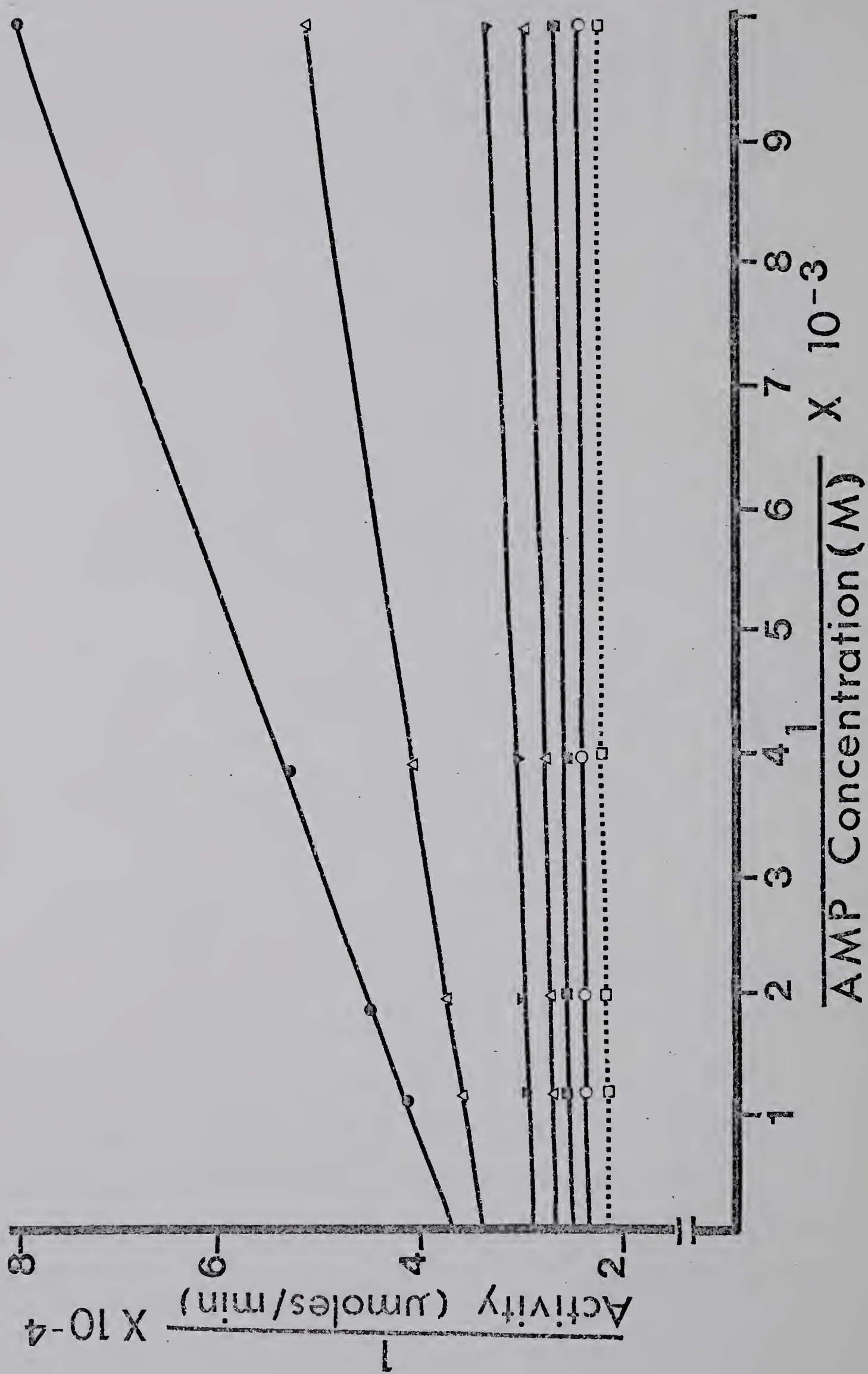


FIGURE 29

LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF
ADENOSINE ON M. SODONENSIS 5'-NUCLEOTIDASE
ACTIVITY

AMP concentrations used ranged from 1×10^{-4} M to 6×10^{-4} M. The ^{14}C -AMP assay technique was used.

□.....□	control
○————○	1×10^{-5} M Adenosine
■————■	2×10^{-5} M Adenosine
△————△	5×10^{-5} M Adenosine
▼————▼	1×10^{-4} M Adenosine
△————△	5×10^{-4} M Adenosine
●————●	2.5×10^{-3} M Adenosine

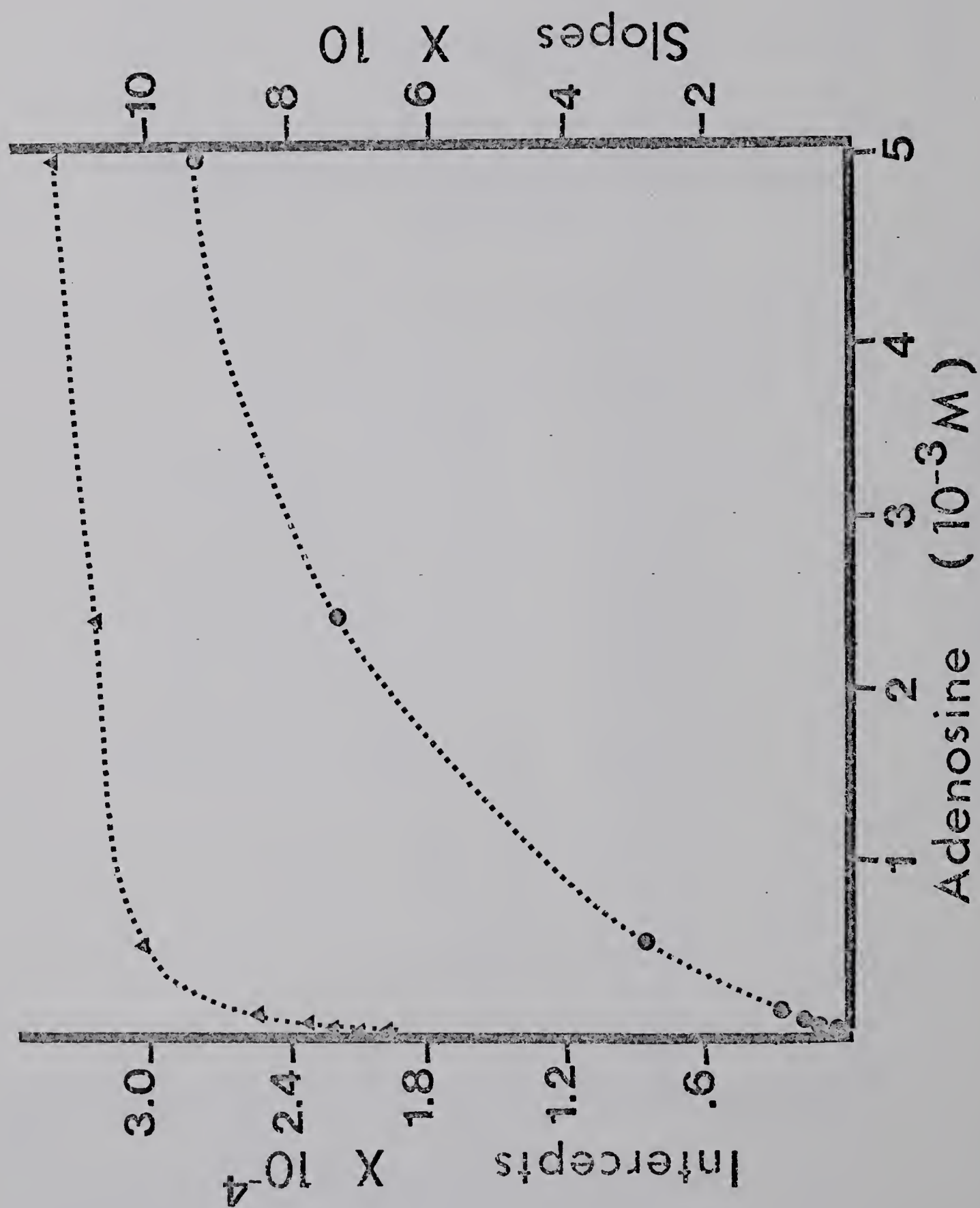


FIGURE 30

REPLOTS OF INTERCEPTS AND SLOPES FROM FIGURE 29
AGAINST ADENOSINE CONCENTRATION

●.....● slopes
▲.....▲ intercepts

Both $\frac{1}{\text{slope}_i - \text{slope}_o}$ and $\frac{1}{\text{intercept}_i - \text{intercept}_o}$ versus

$\frac{1}{I}$ plots gave straight lines (Figure 31). K_i slope equals 3.28×10^{-3} M and K_i intercept equals 5.08×10^{-5} M.

The inhibition of the hydrolysis of other nucleotides by their respective nucleosides was tested in analogous experiments with product (inhibitor) concentrations ranging from 2×10^{-4} to 3×10^{-3} M. (Figures 32 - 36).

The V_{\max} 's were calculated from the vertical intercepts of the Lineweaver-Burk plots. These ranged from 11.5 to 51.4 μ moles of P_i released per mg of protein per hour. In all cases tested, the deoxyribonucleotides had a higher V_{\max} than the corresponding ribonucleotides.

The product inhibition pattern obtained with the various nucleotides was similar to that seen with AMP and adenosine (Figures 32 - 36). The lower nucleoside concentrations gave lines almost parallel to that given by substrate alone. The higher concentrations of inhibitor gave noncompetitive inhibition.

Uridine and cytidine were better inhibitors of their corresponding nucleotides than was adenosine. The other nucleosides were poorer inhibitors. Guanosine, cytidine and uridine gave a greater amount of uncompetitive inhibition than did adenosine.

Replots of slopes and intercepts were not as reliable as those for AMP and adenosine since fewer concentrations of

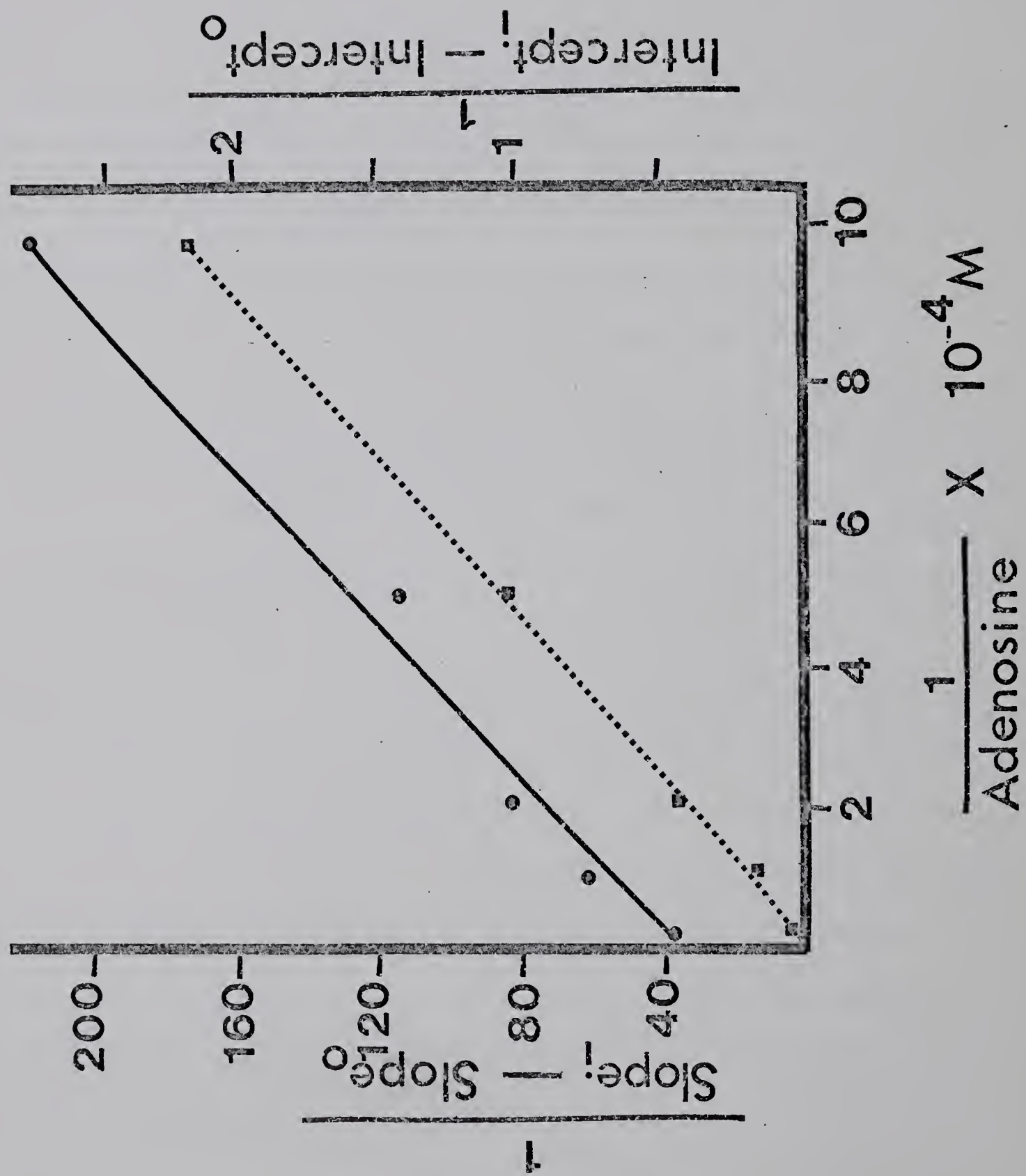
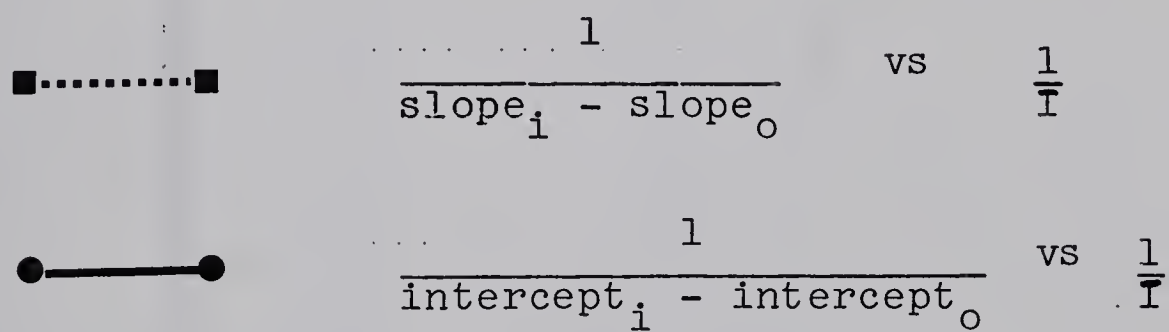


FIGURE 31

REPLOTTING OF RECIPROCAL OF SLOPES AND INTERCEPTS FROM
FIGURE 29 AGAINST RECIPROCAL OF ADENOSINE
CONCENTRATION



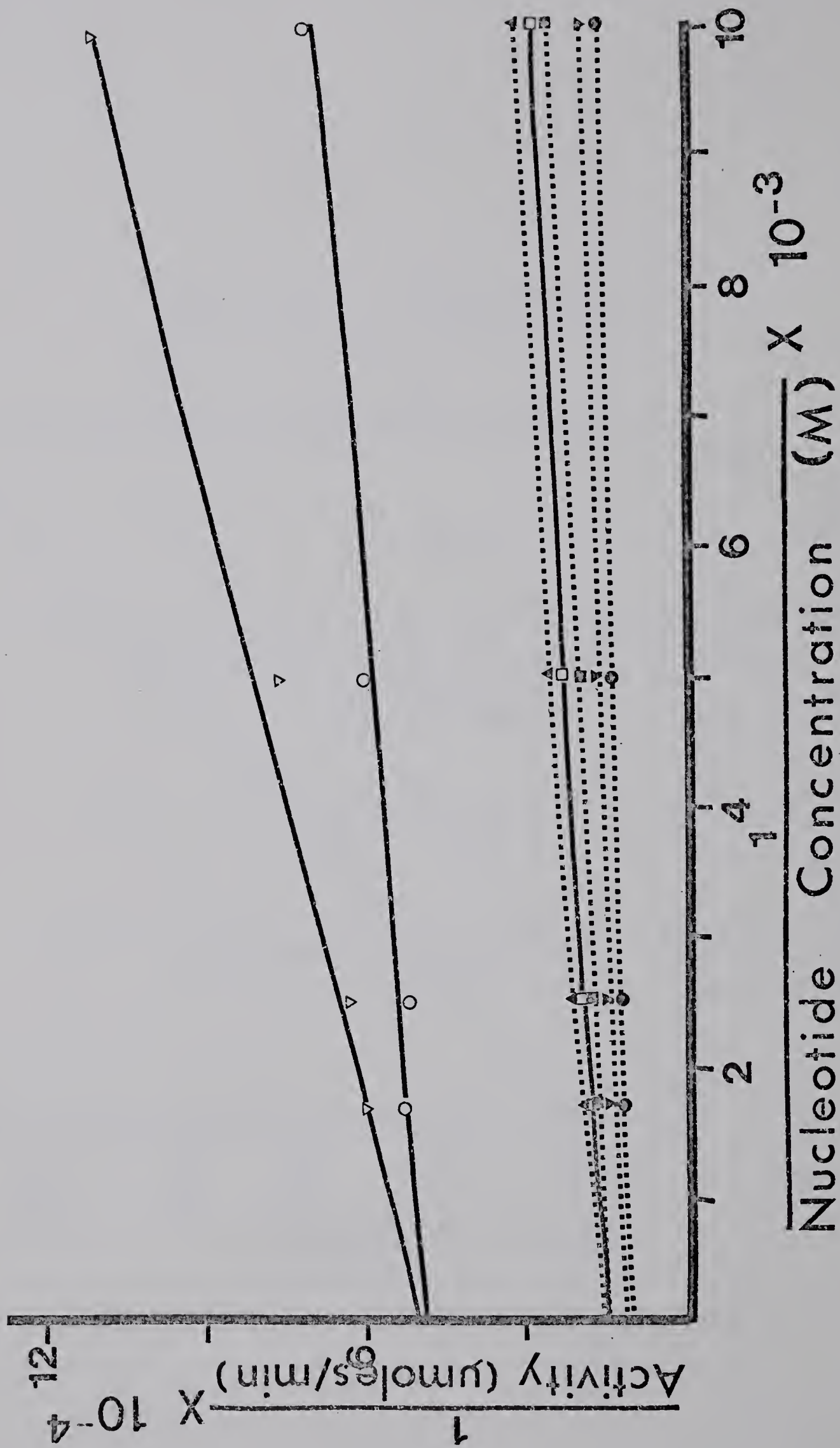


FIGURE 32

LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF CYTIDINE
AND DEOXYCYTIDINE ON THE HYDROLYSIS OF CMP AND dCMP
RESPECTIVELY BY M. SODONENSIS 5'-NUCLEOTIDASE

The substrate concentrations used ranged from 1×10^{-4} M
to 6×10^{-4} M. The P_i released was determined by the
Ames-Dubin technique.

●.....●	control dCMP
▼.....▼	2×10^{-4} M deoxycytidine
■.....■	1×10^{-3} M deoxycytidine
▲.....▲	3×10^{-3} M deoxycytidine
□————□	control CMP
○————○	2×10^{-4} M cytidine
▽————▽	1×10^{-3} M cytidine

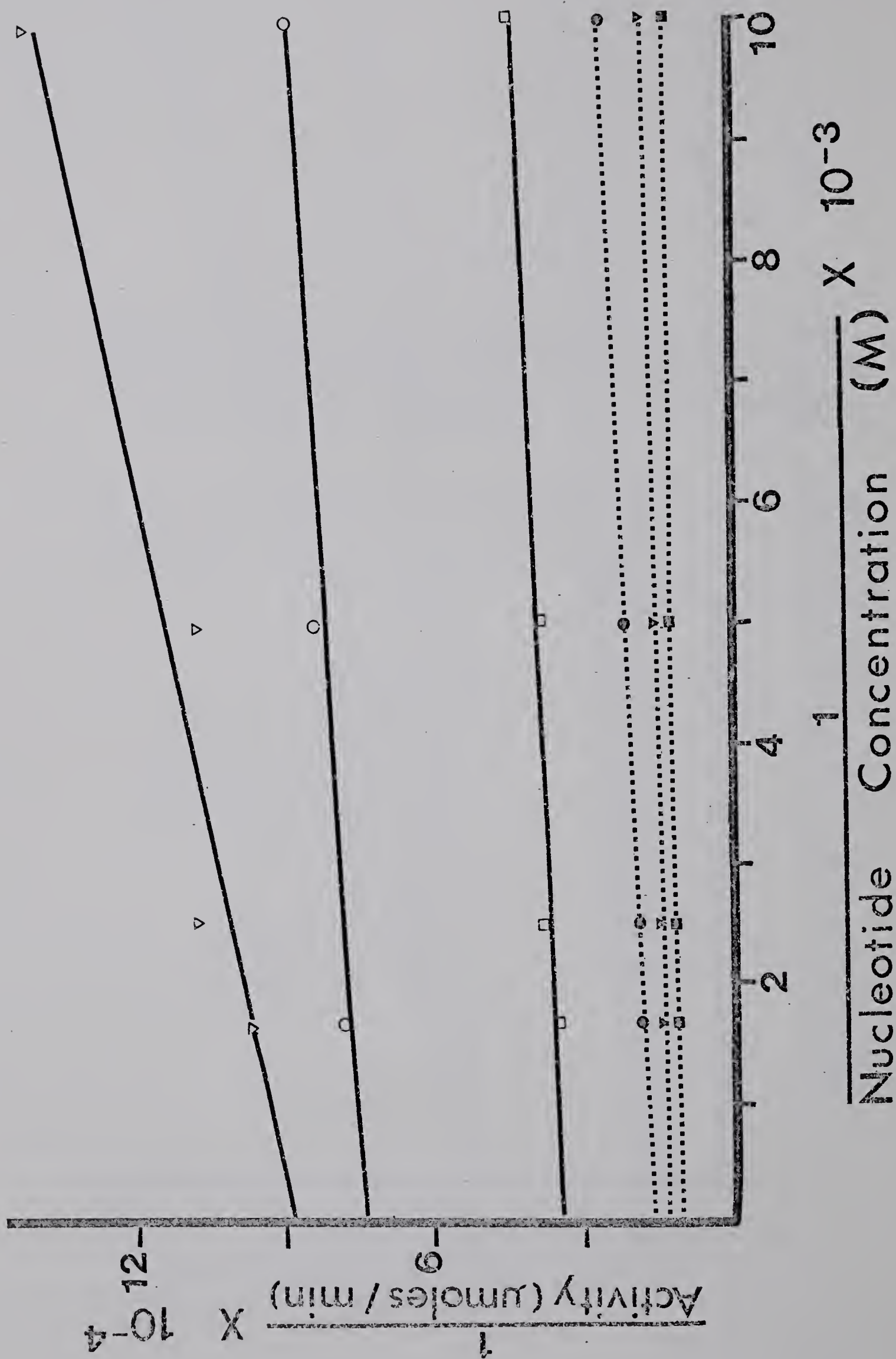


FIGURE 33

LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF URIDINE
AND DEOXYURIDINE ON THE HYDROLYSIS OF UMP AND dUMP
RESPECTIVELY BY M. SODONENSIS 5'-NUCLEOTIDASE

Substrate concentrations and assay procedures as described
for Figure 32.

■.....■	control dUMP
▼.....▼	1×10^{-3} M deoxyuridine
●.....●	3×10^{-3} M deoxyuridine
□————□	control UMP
○————○	2×10^{-4} M uridine
▽————▽	3×10^{-3} M uridine

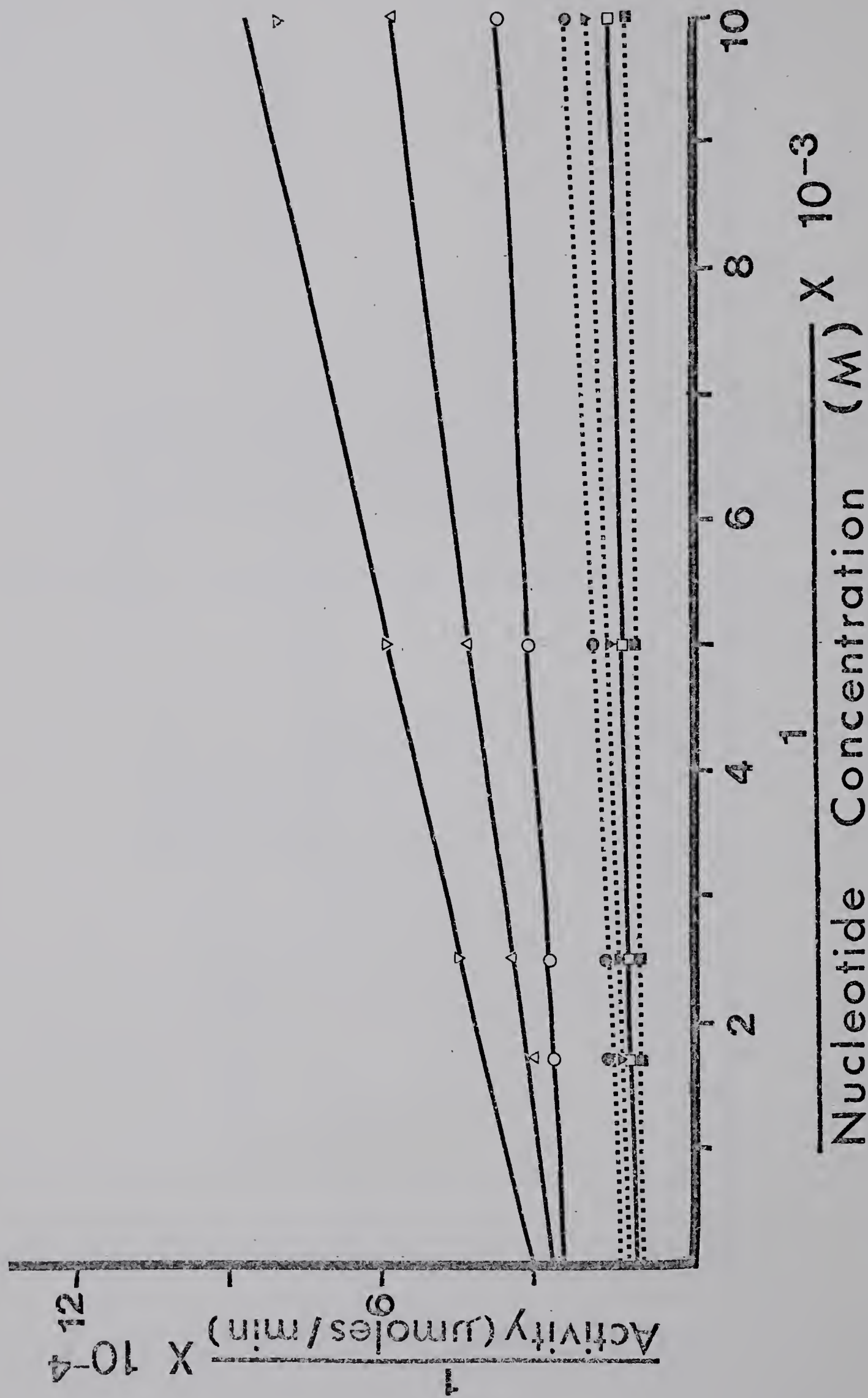


FIGURE 34

LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF ADENOSINE
AND DEOXYADENOSINE ON THE HYDROLYSIS OF AMP AND dAMP
RESPECTIVELY BY M. SODONENSIS 5'-NUCLEOTIDASE

Substrate concentrations and assay procedures as described
for Figure 32.

■.....■	control dAMP
▼.....▼	1×10^{-3} M deoxyadenosine
●.....●	3×10^{-3} M deoxyadenosine
□———□	control AMP
○———○	2×10^{-4} M adenosine
△———△	1×10^{-3} M adenosine
▽———▽	3×10^{-3} M adenosine

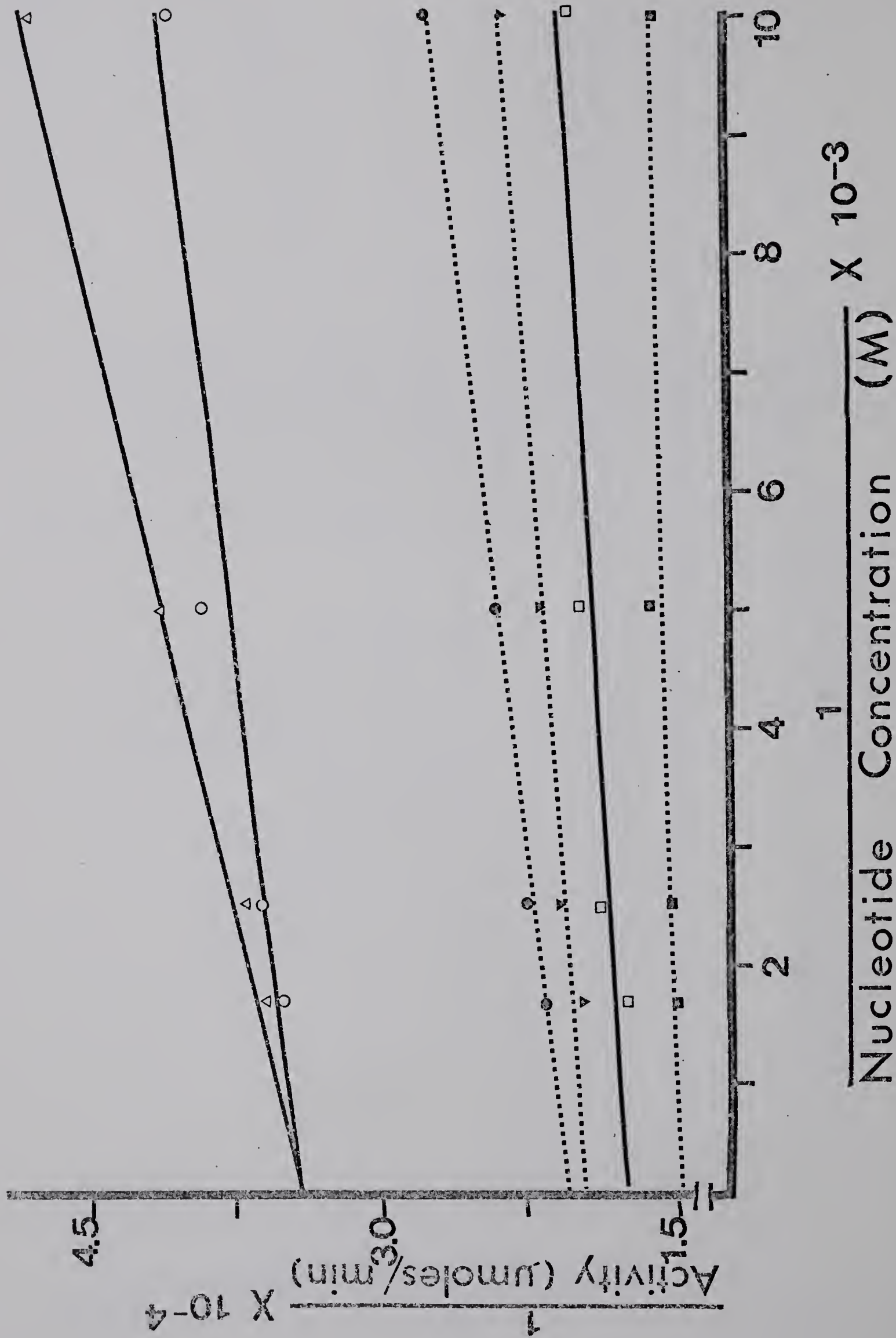


FIGURE 35

LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF GUANOSINE AND DEOXYGUANOSINE ON THE HYDROLYSIS OF GMP AND dGMP RESPECTIVELY BY M. SODONENSIS 5'-NUCLEOTIDASE

Substrate concentrations and assay procedures as described for Figure 32.

■.....■	control dGMP
▼.....▼	1×10^{-3} M deoxyguanosine
●.....●	3×10^{-3} M deoxyguanosine
□————□	control GMP
○————○	5×10^{-4} M guanosine
△————△	1×10^{-3} M guanosine

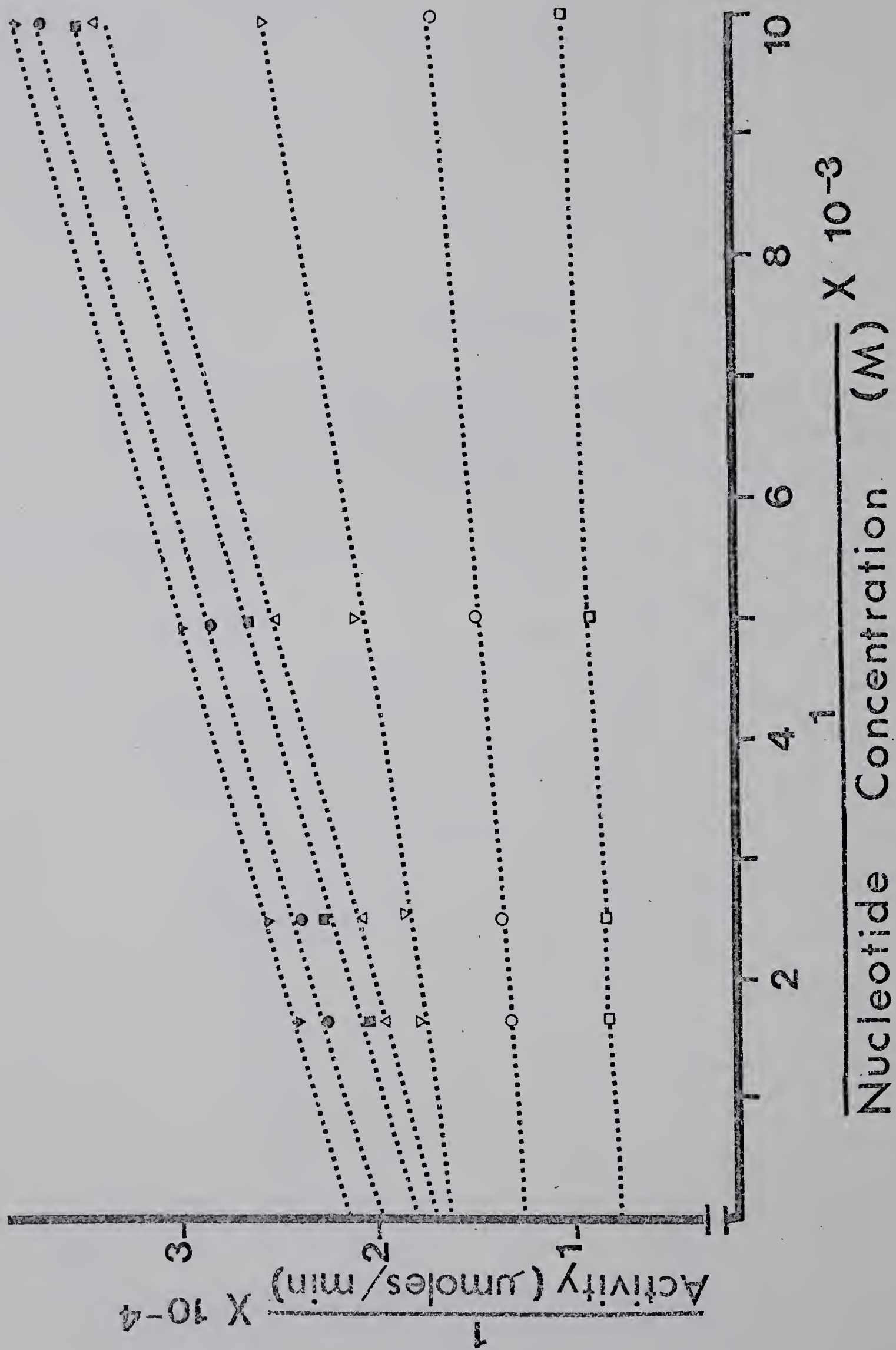


FIGURE 36

LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF INOSINE AND
XANTHOSINE ON THE HYDROLYSIS OF IMP AND XMP RESPECTIVELY
BY M. SODONENSIS 5'-NUCLEOTIDASE

Substrate concentrations and assay procedures as described
for Figure 32.

□.....□	control IMP
○.....○	2×10^{-4} M inosine
▽.....▽	1×10^{-3} M inosine
△.....△	3×10^{-3} M inosine
■.....■	Control XMP
●.....●	2×10^{-4} M xanthosine
▼.....▼	1×10^{-3} M xanthosine

nucleosides were used. In most cases, the replots were similar to those obtained with AMP and adenosine. In some cases, for example, CMP, GMP, UMP and their respective nucleosides, the replots for slopes were almost linear whereas those for the intercepts remained hyperbolic.

5. Effect of Alternative Substrates on AMP Hydrolysis

The effect of the presence of alternative substrates (other nucleoside 5'monophosphates) on the hydrolysis of ^{14}C -AMP was tested by using concentrations of nonradioactive nucleotides ranging from 2×10^{-5} to 1×10^{-4} M with ^{14}C -AMP concentrations of 5×10^{-6} to 4×10^{-5} M (Figures 37-40), in the standard reaction mixture.

The best competitors of ^{14}C -AMP hydrolysis were UMP, CMP, GMP. The corresponding deoxyribonucleotides were all less effective competitors. IMP was about as effective as the deoxyribonucleotides. The inhibition was competitive in some cases. When it was noncompetitive, the slope effects were much greater than the intercept effects. The greatest intercept effects were given by UMP. This same nucleotide had exhibited the greatest degree of uncompetitive inhibition with its corresponding nucleoside.

The difference in effectiveness of the various nucleotides when competing with AMP can be accounted for by their different affinities for the enzyme. The V_{max} 's of

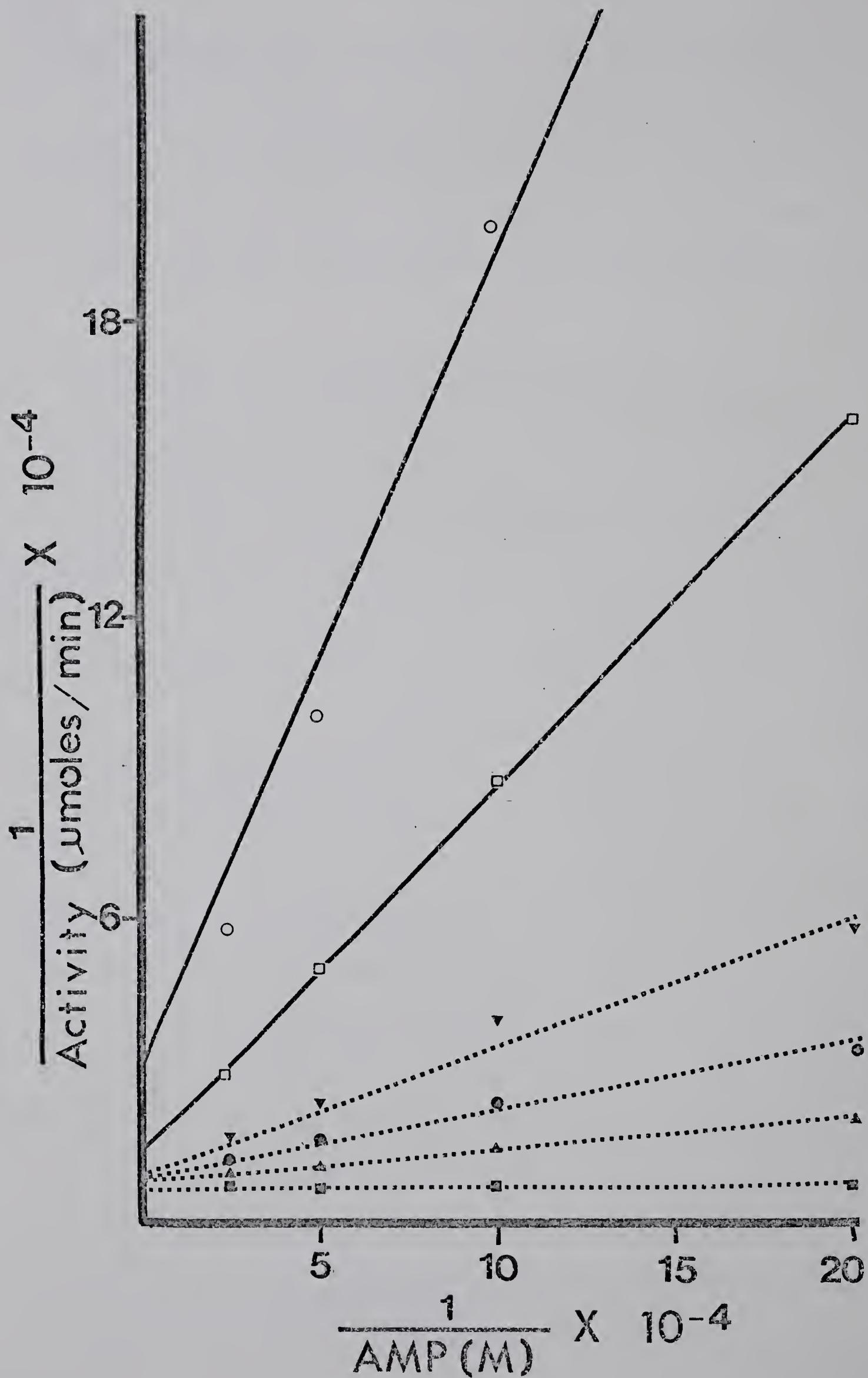


FIGURE 37

LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF UMP AND dUMP
ON THE HYDROLYSIS OF ^{14}C -AMP BY M. SODONENSIS
5'-NUCLEOTIDASE

■.....■	control
▲.....▲	2×10^{-5} M dUMP
●.....●	5×10^{-5} M dUMP
▼.....▼	1×10^{-4} M dUMP
□————□	5×10^{-5} M UMP
○————○	1×10^{-4} M UMP

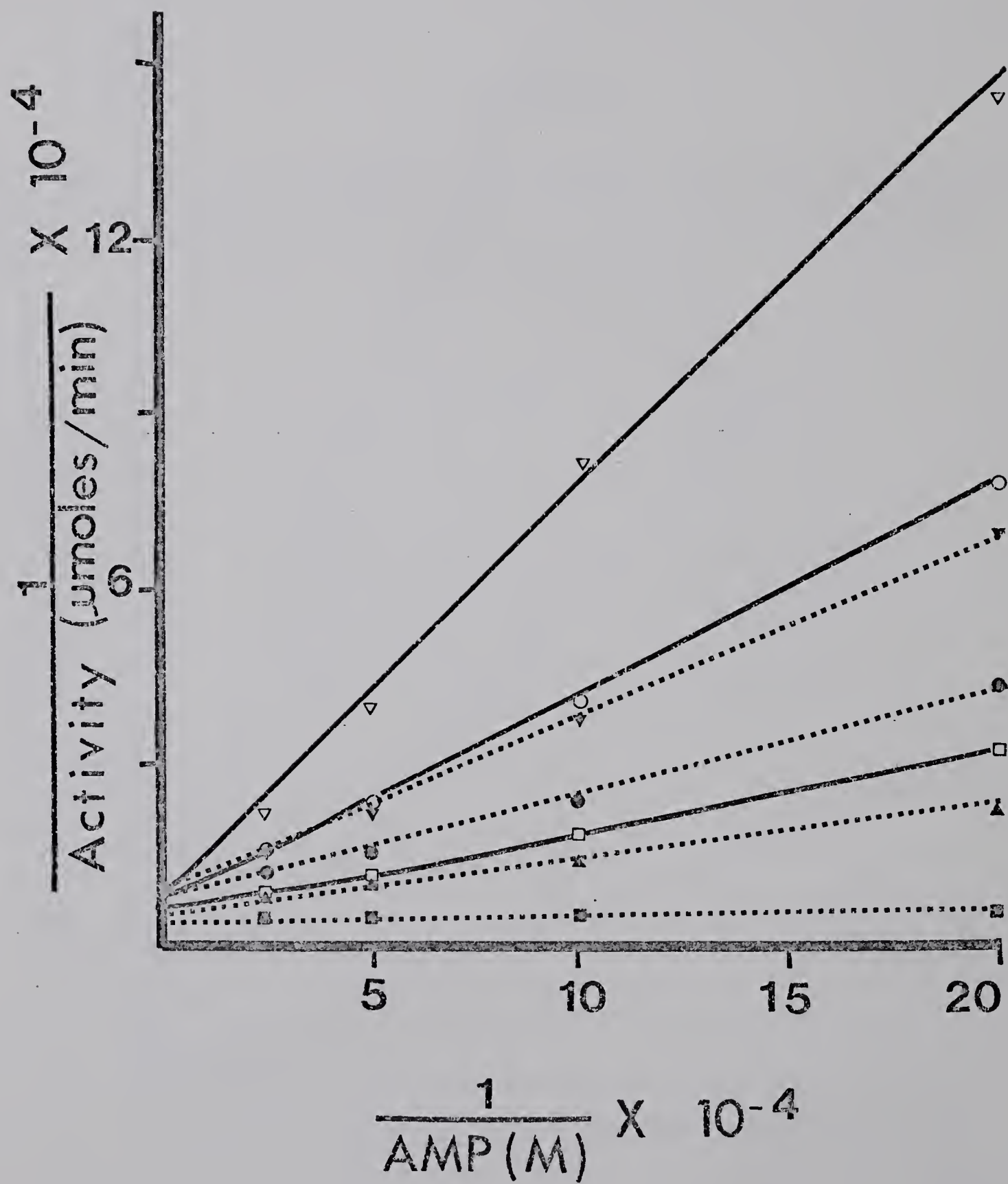
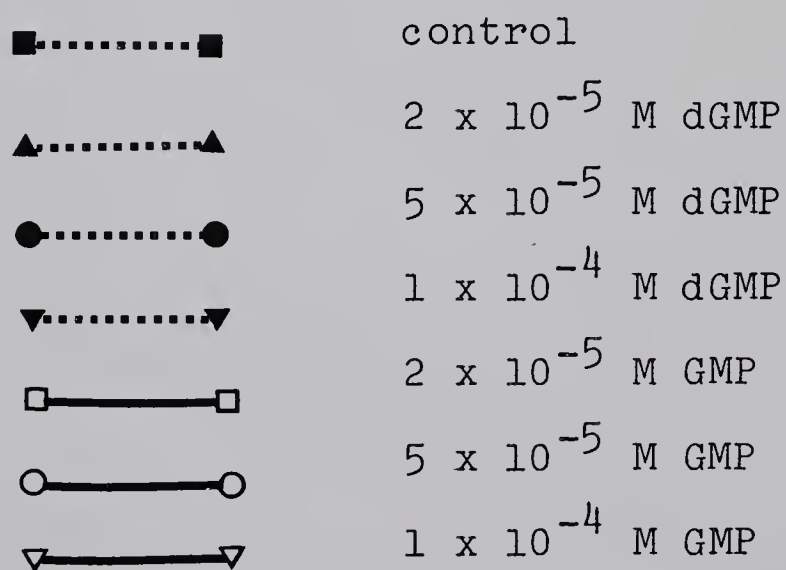


FIGURE 38

LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF GMP AND dGMP
ON THE HYDROLYSIS OF ^{14}C -AMP BY M. SODONENSIS
5'-NUCLEOTIDASE



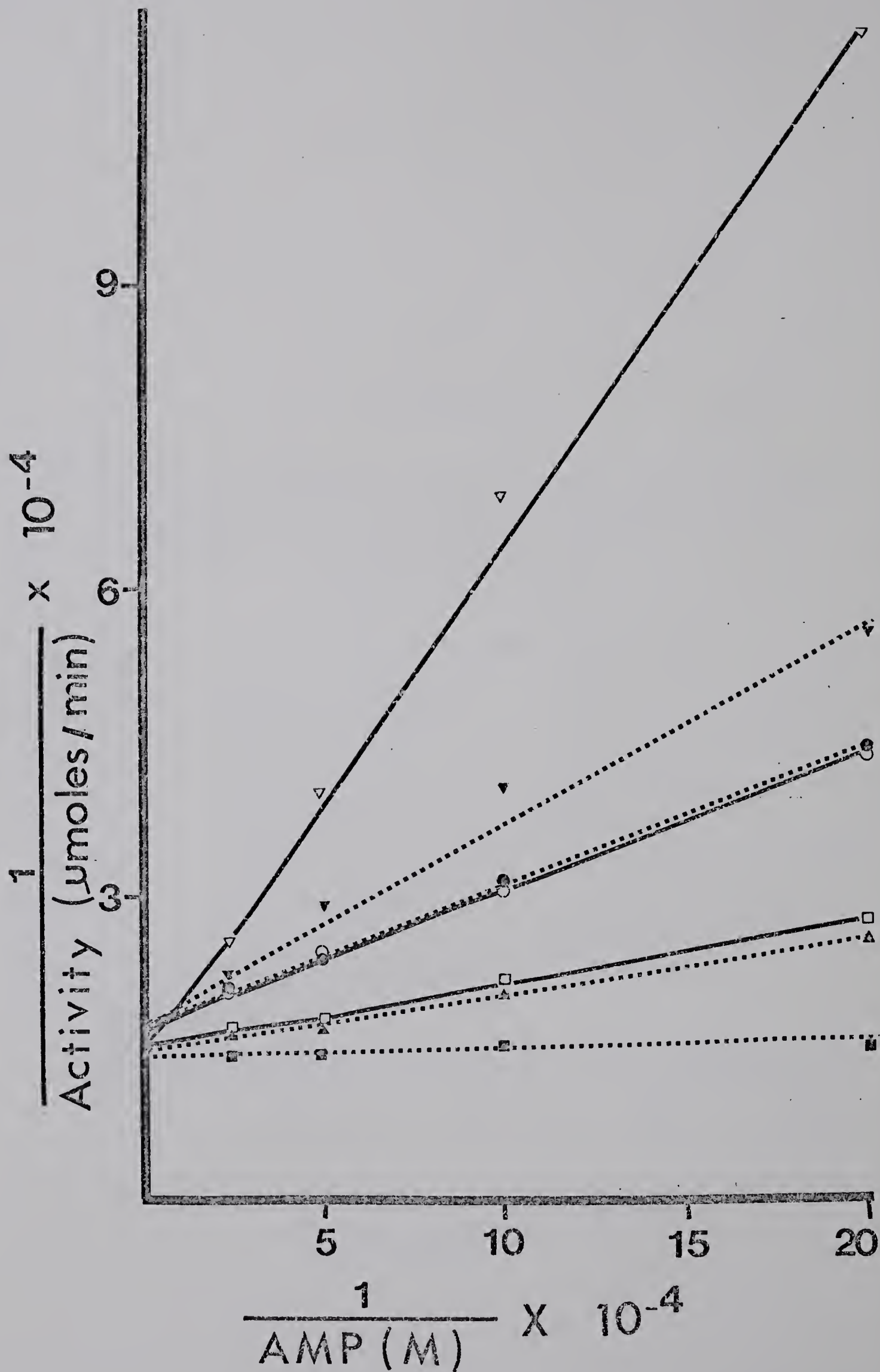


FIGURE 39

LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF CMP AND dCMP
ON THE HYDROLYSIS OF ^{14}C -AMP BY M. SODONENSIS
5'-NUCLEOTIDASE

■.....■	control
▲.....▲	2×10^{-5} M dCMP
●.....●	5×10^{-5} M dCMP
▼.....▼	1×10^{-4} M dCMP
□————□	2×10^{-5} M CMP
○————○	5×10^{-5} M CMP
▽————▽	1×10^{-4} M CMP

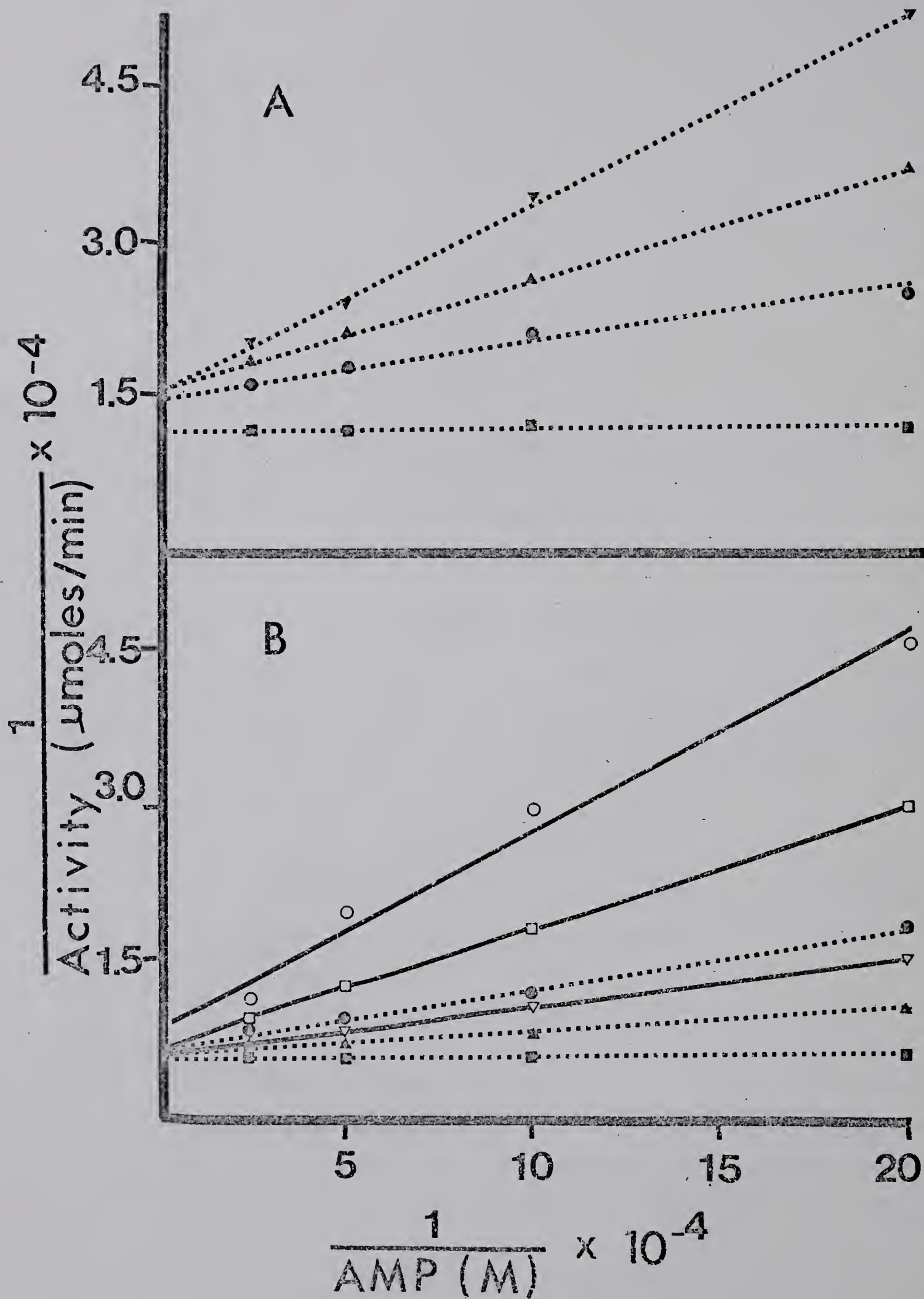


FIGURE 40 A

LINEWEAVER-BURK PLOT SHOWING THE EFFECTS OF dAMP ON THE
HYDROLYSIS OF ^{14}C -AMP BY M. SODONENSIS 5'-NUCLEOTIDASE

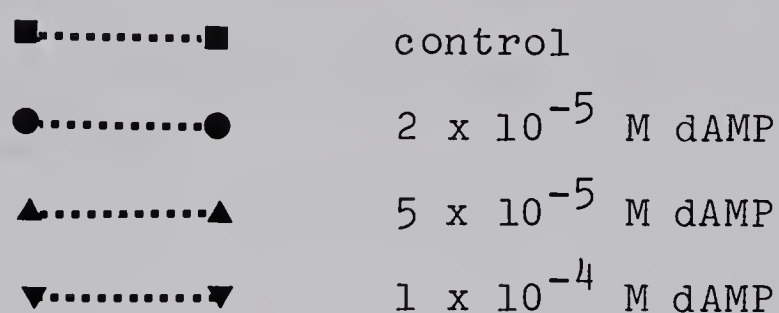
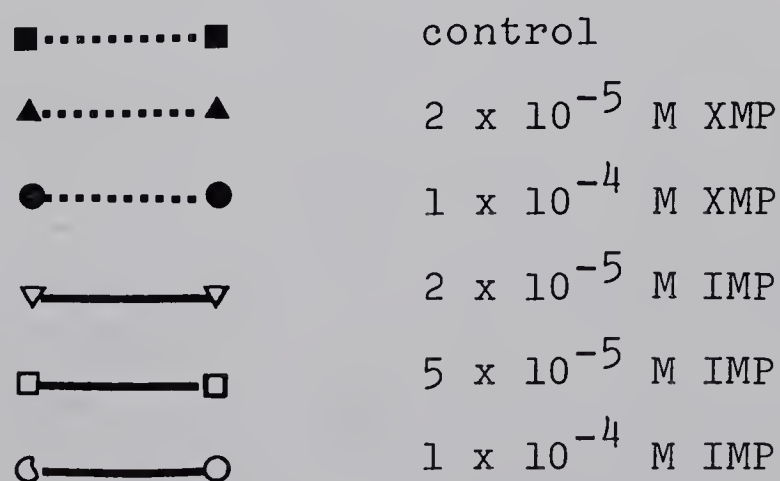


FIGURE 40 B

LINEWEAVER-BURK PLOT SHOWING THE EFFECT OF XMP AND IMP ON
THE HYDROLYSIS OF ^{14}C -AMP BY M. SODONENSIS
5'-NUCLEOTIDASE



the different substrates are quite similar but the degree of binding of the different nucleosides (and, therefore, presumably, of the different nucleotides) to the enzyme varies to a great extent.

There is nothing in the results obtained to indicate the presence of different catalytic sites on the enzyme for different nucleotides.

IV DISCUSSION

Several microorganisms are noted for the production of exoenzymes, whether they be surface-bound as in E. coli or extracellular as in some species of Bacillus. At least five extracellular enzymes are excreted by M. sodonensis: an alkaline phosphatase, two diesterases, a 5'-nucleotidase and a proteinase.

The presence of at least two forms of diesterase has been noted. The production of more than one extracellular RNase or DNase by the same organism is quite common among microorganisms. Two RNA-hydrolyzing enzymes were isolated and characterized in culture filtrates of B. subtilis: a neutral ribonuclease (Coleman, 1967; Mandelstam, 1967) and a phosphodiesterase, degrading also DNA (Okazaki et al, 1966). Ribonucleases with different specificities were isolated from Ustilago sphaerogena (Rushizky et al, 1970). Two ribonucleases with similar or identical amino acid and carbohydrate composition and specificity have been isolated from the culture medium of Rhizopus oligosporus (Woodroof and Glitz, 1971).

On the other hand, the presence of two forms of diesterase in M. sodonensis could be due to the action of the proteinase on the enzyme. Proteolytic modification of an enzyme without loss of activity has been documented in

other instances (Klenow and Overgaard-Hansen, 1970; Bryan, 1970). Highly purified preparations of E. coli alkaline phosphatase are found to contain several distinct enzymatically active forms when analyzed by starch-gel electrophoresis even though these preparations appear homogeneous when examined in the analytical ultracentrifuge (Malamy and Horecker, 1946b; Schlesinger and Anderson, 1968). The various bands have been shown to be controlled by the single phosphatase locus and represent non-genetic modifications of the protein (Signer, Torriani, and Levinthal, 1961). Lazdunski and Lazdunski (1966) proposed that the isoenzymes resulted from the degradation of one of the subunits of the alkaline phosphatase by a proteolytic enzyme. Purification of the proteinase and study of its action on purified diesterase will have to be carried out to determine if such is the case with the M. sodonensis nuclease.

The 5'-nucleotidase and the diesterase of M. sodonensis must be very similar in size and physical characteristics since previous studies (Berry and Campbell, 1967a, b), as well as the present investigation, disclosed that no separation of the two activities could be achieved by gel filtration or analytical ultracentrifugation. Apparently the only difference that can be used to achieve any separation of the two activities is the small charge difference existing between the enzymes.

The four enzyme activities have been shown to be

truly extracellular. Certain conditions of the culture media such as the presence of Tris or EDTA have been shown to cause cell-bound enzymes to be released into the media (Garrard, 1971). However, this does not seem to be the case here. Modifications of the culture media did not result in any change in the relative amounts of cell-bound and free enzymes.

Glew and Heath (1971b) reported that protoplasts of M. sodonensis secreted normal amounts of alkaline phosphatase. Although they had found that lysozyme can release cell-bound enzyme, they concluded from their protoplast studies that the cell wall is not an obligatory component of the secretion process since lysozyme-prepared, sucrose-stabilized protoplasts are capable of extracellular enzyme production. However, the 'protoplasts' were not examined to determine the degree of solubilization of cell wall material achieved by lysozyme with this organism. Lysozyme was found to solubilize only 50% of the cell wall of M. sodonensis in some cases and further observations suggested that the native cell wall is composed of at least two interwoven peptidoglycan nets, one of which is resistant to lysozyme because of extensive peptide cross-bridging and increased levels of O-acetylation (Johnson, 1971). Therefore the normal release of extracellular enzymes of M. sodonensis by such 'protoplasts' does not preclude the role of the cell wall as the site of localization and release of these

extracellular enzymes. An histochemical technique employing deposition of lead indicated that the 5'-nucleotidase activity was associated only with the cell walls and not the periplasmic space or cell membrane (Johnson, 1971).

The study of the effect of phosphate on the production of the extracellular enzymes of M. sodonensis revealed that the control of the alkaline phosphatase production by P_i is different from or more stringent than that exerted over the diesterase and the nucleotidase. The repression of diesterase and 5'-nucleotidase production by P_i can be reversed in part by the added nutrients found in TCS broth or in the Neopeptone; whereas, the alkaline phosphatase remains completely or almost completely repressed in spite of the added nutrients. Moreover, the alkaline phosphatase is fully repressed by the addition of 2% casamino acids to the defined medium whereas the other 2 enzymes are produced in normal amounts.

A mutant was obtained which under certain conditions produces no diesterase and under others, reduced amounts of this enzyme. The levels of the other enzymes are not significantly altered from those of the wild type. Gel electrophoresis of the supernatants indicates that the production of diesterase protein is reduced suggesting that the mutation alters the production of one enzyme protein without affecting the others.

Both the results obtained with the alkaline phos-

phatase and with the diesterase-deficient mutant indicate that the 5'-nucleotidase, the alkaline phosphatase and the diesterase of M. sodonensis are not produced in a pleiotropic fashion. The level of one of the enzymes can be changed without effecting a corresponding change in the levels of the other two enzymes.

The proteinase production is not affected by conditions that significantly change the levels of the other enzymes, such as the addition of P_i or Neopeptone to synthetic medium. This indicates that the control of production of this enzyme is also independent from that of the other enzymes. This finding contrasts with the results obtained with spore-forming species of Bacillus where production of extracellular products is co-regulated and lost or regained in a single mutational event (Schaeffer, 1969).

Unlike the proteinases produced by a number of microorganisms, the proteinase of M. sodonensis does not appear to be subject to catabolite repression or to end product repression. The same level of enzyme is produced by M. sodonensis regardless of the medium used. M. sodonensis may be similar to the Micrococcus sp. studied by McDonald and Chambers (1966) in which amino acids stimulated growth and proteinase production rather than repressing them. However, M. sodonensis requires an organic source of nitrogen for growth (Campbell, et al, 1961) and therefore it is impossible to grow the organism in the absence of amino acids

in order to determine if its proteinase production is also controlled by a form of end-product induction as was the case with other Micrococcus species.

The release of glycoprotein by organisms has been observed in other instances (Semeriva et al, 1969; Hishimura and Nomura, 1959). The diesterase and the 5'-nucleotidase produced by cells grown on TCS broth proved to be glycoproteins consisting of 20% carbohydrate covalently linked to the protein moiety (Berry and Campbell, 1970). Whether there is a release of additional glycoprotein devoid of enzymatic activity in TCS broth has not been determined but the relative ease with which enzyme preparations giving a single peak on ultracentrifugation are obtained from TCS broth as compared to the preparations from the synthetic medium suggests that a similar glycoprotein fraction is absent or much smaller in the TCS broth. This difference is probably correlated with the marked differences noted between the cell walls of the cells grown in TCS broth and those of cells grown in the synthetic medium. The latter had twice as many hexosamine residues as the former and a much less complex peptidoglycan organization (Johnson, 1971).

The extracellular alkaline phosphatase of M. sodonensis has been purified to homogeneity as judged by polyacrylamide gel electrophoresis and sedimentation velocity analysis. The molecular weight determined by molecular sieve chromatography is about 55,000 daltons.

The M. sodonensis alkaline phosphatase has a broad substrate specificity and hydrolyzes a wide variety of phosphorylated substrates at rates comparable to that observed for AMP. This has also been observed for the alkaline phosphatases of E. coli (Garen and Levinthal, 1960; Heppel, Harkness, and Hilmo, 1962) and Pseudomonas fluorescens (Friedberg and Avigad, 1967). Glew and Heath (1971a) observed that the alkaline phosphatase of M. sodonensis hydrolyzes pyrophosphates and nucleotide triphosphates.

Calcium is required for stabilization of the enzyme as well as for expression of catalytic activity. Incubation with EDTA results in prompt and complete inactivation of the enzyme. Restoration of full activity to the EDTA-treated enzyme is effected by calcium. Chelating agents such as o-phenanthroline, 8-hydroxyquinoline, cyanide and cysteine, which are potent inhibitors of the zinc-containing alkaline phosphatase of E. coli (Plocke; Levinthal and Vallee, 1962), have no effect on the alkaline phosphatase of M. sodonensis or produce an effect only when used in higher concentrations than those employed with the E. coli alkaline phosphatase. In this respect, the alkaline phosphatase of M. sodonensis is similar to that of Pseudomonas fluorescens (Friedberg and Avigad, 1967).

Glew and Heath (1971a) have determined that the M. sodonensis alkaline phosphatase contains a minimum of 8 gram atoms of calcium per mole of enzyme, and that this

calcium is firmly bound to the protein, the binding constant being in the order of 1.5×10^{-5} M.

The Michaelis constant, K_m , for PNPP is 2.4×10^{-5} M, close to that obtained for the E. coli alkaline phosphatase (Garen and Levinthal, 1960). The M. sodonensis alkaline phosphatase, like the E. coli enzyme (Garen and Levinthal, 1960; Torriani, 1960), is sensitive to product inhibition by inorganic phosphate. The P_i inhibition is competitive in character and the K_i was estimated at 3.5×10^{-4} M when PNPP was employed as substrate. With AMP as substrate, P_i gives a biphasic inhibition pattern with a higher inhibition constant occurring at lower substrate concentrations. With E. coli alkaline phosphatase, it was found that the Lineweaver-Burk plot also gave a biphasic pattern (Heppel, Harkness and Hilmoie, 1962). Measurements with low substrate concentrations yielded a K_m of 1.4×10^{-5} M for PNPP and 3.3×10^{-5} M for AMP. With substrate concentrations of 1×10^{-3} to 3×10^{-2} , the K_m 's were 3.0×10^{-3} M for PNPP and 1.1×10^{-3} M for AMP. This phenomenon was also encountered with the alkaline phosphatase of Pseudomonas fluorescens (Friedberg and Avigad, 1967). Also, in the native E. coli alkaline phosphatase, there are two P_i -binding sites that differ in apparent dissociation constant; the magnitude of this difference depends upon the ionic strength of the solvent and the pH (Simpson and Vallee, 1970). Although in the case of M. sodonensis alkaline phosphatase, a biphasic Lineweaver-

Burk plot is not found, a similar effect could account for the biphasic product inhibition pattern.

In contrast to the E. coli enzyme for which P_i and arsenate are equally effective inhibitors (Garen and Levinthal, 1960), the M. sodonensis enzyme was much more sensitive to arsenate than it was to P_i .

The alkaline phosphatase of M. sodonensis possesses transphosphorylase activity as demonstrated by the ability of the enzyme to transfer phosphate from PP_i or AMP to glucose to form glucose-6-phosphate. This activity is also associated with the alkaline phosphatase from E. coli (Anderson and Nordlie, 1967). It has been found that enzyme activity increases with the concentration of Tris buffer in the case of both the E. coli and the M. sodonensis alkaline phosphatases. Dayan and Wilson (1964) determined that the amount of p-nitrophenol produced exceeds the amount of P_i when p-nitrophenol phosphate is hydrolyzed by E. coli alkaline phosphatase in the presence of Tris due to the transphosphorylation of the Tris. The rate of utilization of substrate is increased in the presence of Tris, indicating that the dephosphorylation of the enzyme is the rate-controlling step.

The reaction of the alkaline phosphatase from M. sodonensis with sulfhydryl reagents indicates that a sulfhydryl group is necessary for enzymatic activity. This

was confirmed by measurement of the ^{14}C -CMB uptake by M. sodonensis alkaline phosphatase (Glew and Heath, 1971a). The authors concluded that the native enzyme possesses an accessible and relatively active sulfhydryl group which is required for full enzymatic activity.

The kinetic study of the 5'-nucleotidase of M. sodonensis indicated that both products of the hydrolytic reaction, the nucleoside and the P_i , interacted with the enzyme. The binding of these products to the enzyme was detected by equilibrium dialysis of the nucleoside with the enzyme, by the elution of $^{32}\text{P}_i$ with the enzyme on gel filtration, by protection from inactivation, by heat or by protein reagents, afforded by both of the hydrolysis products.

There is some indication that P_i and the nucleosides do not interact with the enzyme at the same site since each product protected the 5'-nucleotidase from inactivation by different protein reagents and P_i does not inhibit the binding of adenosine to the enzyme.

P_i appeared to be a competitive inhibitor of the enzymatic reaction with some nonspecific or 'dead-end' inhibition occurring with certain enzyme preparations or at higher substrate concentrations. It is interesting to note that the older enzyme preparations that manifested a loss of sensitivity to P_i inhibition had concurrently acquired a requirement for Mg^{2+} for activity, whereas the 5'-nucleotidase normally is active in its absence. The strong binding

of P_i to many cations may cause changes in cation concentrations in the enzymatic assay and thus account for some of the P_i inhibition.

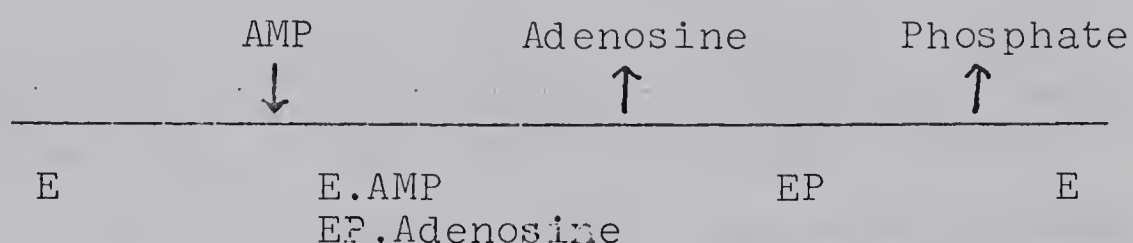
The hyperbolic replots for nucleoside inhibition would seem to indicate alternative binding sequences in the mechanism, either partial dead-end binding or a random mechanism. The uncompetitive inhibition noted in the product inhibition patterns may indicate dead-end binding of adenosine to some enzyme form. This would create an alternative reaction sequence and thus a partially random mechanism. Terms containing I would occur both in the numerator and in the denominator of slopes or intercepts equations. Replots of slopes or intercepts in this case would give hyperbolas with

$$\text{slope or intercept} = \frac{a + bI}{c + bI} \quad \text{where } bc > ad.$$

Such dead-end binding may also account for the slight intercept effects noted with some alternative substrates. Alternative substrates competing for the same site on the enzyme should give competitive inhibition. It is significant that intercept effects occurred with those nucleotides that showed the strongest affinity for the enzyme.

Several hydrolytic reactions which appear to be Ping Pong Bi Bi with water as the second substrate, give apparent ordered Uni Bi kinetics in aqueous solution where the water concentration is constant (Cleland, 1963). The

kinetic data obtained indicate that such may be the case with M. sodonensis 5'-nucleotidase. The following reaction sequence



would result in competitive inhibition of AMP hydrolysis by P_i and noncompetitive inhibition by adenosine. The presence of dead-end binding or some other non-specific inhibition by the two products does not permit unreserved adoption of this reaction sequence until the system is studied in more detail.

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